University of Alberta

CHARACTERIZATION AND GENETIC ANALYSIS OF ENTEROCIN B PRODUCTION AND IMMUNITY IN *Enterococcus faecium* BFE 900

by

Charles M. A. P. Franz

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Food Microbiology

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Characterization and Genetic Analysis of Enterocin B Production and Immunity in *Enterococcus faecium* BFE 900** submitted by **Charles Marie Antoine Paul Franz** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Food Microbiology**.

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ABSTRACT

Class II bacteriocins are small, heat stable peptides that undergo minimal posttranslational modification that is generally limited to cleavage of a leader peptide. Export of most class II bacteriocins depends on dedicated secretion proteins, while a few are exported by the general secretion (*sec*) pathway. Immunity of the producer to its own bacteriocin depends on the product of an immunity gene, usually located downstream of the bacteriocin structural gene in the same operon. *Enterococcus faecium* BFE 900 isolated from black olives produced the heat stable bacteriocin enterocin B. Enterocin B was purified and its amino acid sequence determined. It consists of 53 amino acids and shares homology with carnobacteriocin A. Enterocin B and carnobacteriocin A are classified as class IIa bacteriocins, based on their anti-*Listeria* activity; however, they differ from these bacteriocins because they do not contain a YGNGYXC consensus motif at the N terminus. The enterocin B structural gene was detected on a 2.2-kb and a 12.0-kb chromosomal fragment. Both fragments were cloned and sequenced. Genetic analysis showed that enterocin B is produced as a prebacteriocin, with a 'double-glycine-type' leader peptide. Apart from an atypically-located immunity gene, no other genes associated with bacteriocin production were identified from the chromosomal fragments. This differs from other bacteriocin systems, where bacteriocin structural, immunity, transport and possibly regulatory genes are located in a gene cluster. Enterocin B was expressed in heterologous hosts by both the bacteriocin dedicated pathway and the *sec*-pathway. The immunity genes for enterocin B and carnobacteriocin A were located downstream and in opposite orientation to the bacteriocin structural genes and their protein products shared

considerable homology. Absence of the YGNGVXC-consensus motif and the atypical arrangement of the immunity genes for enterocin B and carnobacteriocin A may suggest a new class of bacteriocins, class IId. The identification of genes for enterocin B and carnobacteriocin A production and immunity in this study allows incorporation of these genes into multiple bacteriocin cassettes in a food grade vector. Use of LAB starters which contain such a vector is envisaged for biopreservation of foods.

DEDICATION

In memory of my mother, Carla Franz-Frissen

(11. 12. 1939 – 15. 02. 1996)

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TABLE OF CONTENTS

1. Introduction and literature review.....1

1.1. The lactic acid bacteria.....1

1.1.1. Phylogeny and taxonomy of lactic acid bacteria.....1

1.1.2. Lactic acid bacteria as food spoilage organisms.....2

1.1.3. Lactic acid bacteria in food preservation.....3

1.2. Bacteriocins produced by lactic acid bacteria.....5

1.2.1. Definition and classification of bacteriocins.....5

1.2.2. Genetics of bacteriocin production.....7

1.2.2.1. The bacteriocin and its gene.....8

1.2.2.2. The immunity protein and its gene.....11

1.2.2.3. Dedicated bacteriocin transport: ABC transporter and accessory protein genes.....14

1.2.2.4. Bacteriocin transport by the bacterial preprotein translocase.....15

1.2.3. Regulation of class II bacteriocin production.....18

1.2.4. Mode of action of class II bacteriocins.....24

1.2.5. Applications of class II bacteriocins from lactic acid bacteria in food.....25

1.3. The enterococci.....28

1.3.1. Phylogeny and taxonomy of enterococci.....28

1.3.2. Environmental sources and food contamination.....29

1.3.3. Enterococci in the food supply.....30

1.3.3.1. Association of enterococci with meats.....30

1.3.3.2. The role of enterococci in cheese manufacture.....32

1.3.4. Enterococcal bacteriocins: the enterocins.....34

1.3.5. Enterococci as probiotics.....37

1.3.6. Enterococci in disease.....39

1.3.7. Antibiotic resistance.....40

1.3.8. Gene transfer mechanisms in enterococci.....42

1.3.9. Enterococcal virulence factors.....43

1.3.9.1. Colonization....43
1.3.9.2. Adherence....44
1.3.9.3. Translocation....45
1.3.9.4. Resistance to host defense mechanisms....45
1.3.10. Pathology of enterococcal infection....46
1.4. Implications of enterococci in foods....48
1.5. Research objective....53
1.6. References....54
2. Isolation and identification of a bacteriocin producing *Enterococus faecium* from black olives, and characterization of its bacteriocin....78
2.1. Introduction....78
2.2. Methods and Materials....80
2.2.1. Isolation of lactic acid bacteria....80
2.2.2. Screening for bacteriocin producing LAB isolates....80
2.2.3. Cultures and media....81
2.2.4. Identification of bacteriocin-producing isolates....81
2.2.5. Partial purification, activity assay and spectrum of activity....82
2.2.6. Effect of heat, enzymes and pH on bacteriocin activity....83
2.2.7. Bactericidal activity....83
2.2.8. Bacteriocin production kinetics....84
2.2.9. Effect of pH on bacteriocin activity....84
2.2.10. Influence of medium pH and components on bacteriocin production....84
2.2.11. Plasmid DNA isolation....85
2.3. Results....86
2.3.1. Identification of bacteriocin-producing LAB....86
2.3.2. Effect of enzymes on the antibacterial activity of neutralized supernatant....88
2.3.3. Bactericidal activity of neutralized supernatant....88
2.3.4. Effect of heat and pH on bacteriocin activity....90

2.3.5. Spectrum of activity.....91
2.3.6. Bacteriocin production kinetics.....91
2.3.7. Influence of medium pH and components on bacteriocin production.....92
2.3.8. Plasmid isolation.....94
2.4. Discussion.....95
2.5. References.....101
3. Atypical genetic locus for enterocin B production in *Enterococcus faecium* BFE 900.....106
3.1. Introduction.....106
3.2. Methods and Materials.....108
3.2.1. Bacterial strains and culture conditions.....108
3.2.2. Bacteriocin activity assays and induction.....108
3.2.3. Enterocin B purification and Tricine-SDS-PAGE electrophoresis.....110
3.2.4. N-terminal amino acid sequencing and mass spectrometry.....111
3.2.5. DNA isolation, manipulation and hybridization.....112
3.2.6. DNA and amino acid sequence analysis.....113
3.2.7. Nucleotide sequence accession number.....113
3.2.8. PCR amplification.....113
3.2.9. Expression of enterocin B structural and immunity genes in heterologous hosts.....114
3.2.10. Tests for multiple bacteriocin production.....115
3.3. Results.....116
3.3.1. Induction.....116
3.3.2. Enterocin B purification and mass spectral analysis.....116
3.3.3. Nucleotide sequence and identification of the enterocin B structural gene.....120
3.3.4. Amino acid homology.....123
3.3.5. Heterologous expression of enterocin B.....125
3.3.6. Multiple bacteriocin production.....126

3.3.7. Immunity gene.....128
3.4. Discussion.....129
3.5. References.....135
4. Identification of the gene encoding immunity to carnobacteriocin A in *Carnobacterium piscicola* LV17A.....140
4.1. Introduction.....140
4.2. Methods and materials.....142
4.2.1. Bacterial strains and culture conditions.....142
4.2.2. Deferred inhibition tests and bacteriocin activity assays.....144
4.2.3. DNA isolation, manipulation and electroporation.....144
4.2.4. Localization of the carnobacteriocin A immunity gene.....145
4.2.5. PCR amplification and cloning of the carnobacteriocin A immunity gene.....145
4.2.6. Homologous expression of carnobacteriocin A immunity.....147
4.3. Results.....147
4.3.1. Identification of the carnobacteriocin A immunity gene.....147
4.3.2. Effect of promoter strength on homologous expression of immunity.....149
4.4. Discussion.....149
4.5. References.....153
5. General discussion and conclusions.....157
5.1. References.....169

LIST OF TABLES

Table 1.1 Double-glycine-type leader peptides of class II bacteriocins.....10

Table 1.2 Signal peptides associated with class II bacteriocins.....11

Table 1.3 Characteristics of immunity genes of class II bacteriocins.....12

Table 1.4 Amino acid sequences of induction factors and putative induction factors.....22

Table 1.5 Numbers and predominance of *Enterococcus* spp. in cheeses from Mediterranean countries.....33

Table 1.6 Well characterized bacteriocins produced by *E. faecium* and *E. faecalis*.....35

Table 2.1 Media used to determine effect of medium components on bacteriocin production.....85

Table 2.2 Antagonistic activity of neutralized supernatant and partially purified fraction from *E. faecium* BFE 900 against lactic acid and non-lactic acid bacteria.....87

Table 2.3 Effects of enzymes, heat and pH on inhibitory antagonistic activity of cell free supernatant of *Enterococcus faecium* BFE 900 89

Table 2.4 Bactericidal effect of enterocin 900 on *Lactobacillus sake* DSM 20017 in MRS broth at 30°C.....90

Table 2.5 Influence of initial pH of MRS medium on numbers (log CFU ml^{-1}) and bacteriocin production (AU ml^{-1}) of *Enterococcus faecium* BFE 900.....93

Table 2.6 Influence of medium composition on numbers (log CFU ml^{-1}) and bacteriocin production (AU ml^{-1}) of *Enterococcus faecium* BFE 900; determined after growth at 30°C for 24 hours.....94

Table 3.1 Bacterial strains and plasmids used in this study.....109

Table 3.2 Purification of enterocin B produced by *E. faecium* BFE 900 in 3 liters APT broth culture at 30°C.....117

Table 4.1 Bacterial strains and plasmids used in this study.....143

LIST OF FIGURES

Figure 1.1 Schematic representation of bacteriocin secretion by the dedicated-secretion pathway.....8

Figure 1.2 Diagram showing the organization of domains of ABC transporter and accessory proteins.....16

Figure 1.3 Diagram showing transport of class IIc bacteriocins bearing signal peptides by the bacterial preprotein translocase.....18

Figure 1.4 Schematic representation of a model for induction of bacteriocin production mediated by the induction factor and two-component regulatory system.....20

Figure 1.5 Alignment of promoter and regulatory like box sequences from carnobacteriocins, plantaricins and sakacin P and their respective induction factor genes.....23

Figure 2.1 Results of clustering analysis based on protein patterns of *Enterococcus* and *Lactococcus* reference strains and isolates.....88

Figure 2.2 Viable count (log CFU ml^{-1}) and bacteriocin production (AU ml^{-1}) of *Enterococcus faecium* BFE 900 during growth in MRS broth (pH 7.0) at 30°C.....92

Figure 3.1 Tricine-SDS-PAGE gel showing HPLC purified enterocin B and molecular weight markers.....118

Figure 3.2 Electrospray mass spectrum of enterocin B.....119

Figure 3.3 Single stranded nucleotide sequence of a 2.2-kb *Hin*dIII fragment of *E. faecium* BFE 900 chromosomal DNA.....121

Figure 3.4 Organization of genes on the cloned 12.0-kb and 2.2-kb chromosomal fragments from *Enterococcus faecium* BFE 900 associated with enterocin B production.....122

Figure 3.5 Amino acid sequences of double-glycine-type leader peptides.....124

Figure 3.6 Deferred inhibition tests showing heterologous expression of enterocin B.....126
Figure 3.7 Deferred inhibition tests showing multiple bacteriocin production by *E. faecium* BFE 900.....127
Figure 3.8 Deferred inhibition tests showing heterologous expression of immunity to enterocin B 128
Figure 4.1 Organization of genes involved in carnobacteriocin A production and immunity.....146
Figure 4.2 Deferred inhibition tests showing homologous expression of immunity to carnobacteriocin A 148

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
APT	All Purpose Tween
ATP	Adenosine triphosphate
AU	Activity units
Bac^-	Bacteriocin negative
Bac^+	Bacteriocin positive
Bp	Base pair(s)
CAA	Casamino acids
Cbn	Carnobacteriocin
CFU	Colony forming units
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Ent	Enterocin
g	Gram(s)
$g\ l^{-1}$	Gram(s) per liter
h	Hour(s)
HPLC	High performance liquid chromatography
IF	Induction factor
kb	Kilobase(s)
l	Liter
LAB	Lactic acid bacteria

Laf	Lactacin F
Lci	Lactococcin
Lcn	Leucocin
MALDI-TOF	Matrix assisted laser desorption/ionization time-of-flight
MRS	de Man, Rogosa and Sharpe
min	Minute(s)
ml	Millilitre
PAGE	Polyacrylamide gel electrophoresis
Ped	Pediocin
Pln	Plantaricin
RBS	Ribosome binding site
SAB	Sodium acetate buffer
Sak	Sakacin
SDS	Sodium dodecyl sulfate
Sec	Secretory
TFA	Trifluoroacetic acid
vol./vol.	Volume per volume

CHAPTER 1*

INTRODUCTION AND LITERATURE REVIEW.

1.1. The lactic acid bacteria.

1.1.1. *Phylogeny and taxonomy of lactic acid bacteria.*

The gram-positive bacteria are subdivided into two main groups or clusters. One cluster consists of bacteria with a mol% G+C of the DNA higher than 55% which is designated the '*Actinomycetes*' subdivision, or the 'high-GC' subdivision (Axelsson, 1993). This cluster contains the genera *Bifidobacterium, Arthrobacter, Micrococcus, Propionibacterium, Microbacterium, Corynebacterium, Actinomycetes* and *Streptomyces* (Woese, 1987; Stackebrandt and Teuber, 1988). The other cluster is termed the '*Clostridium*' or 'low-GC' subdivision, and contains species with a mol% G+C content in the DNA of 55% or less. All lactic acid bacteria (LAB) are included in this subdivision, together with aerobes and facultative anaerobes such as *Bacillus, Staphylococcus* and *Listeria,* and anaerobes such as *Clostridium, Peptococcus* and *Ruminococcus* (Woese, 1987; Stackebrandt and Teuber, 1988). The LAB are considered to form a 'supercluster' which phylogenetically lies in between the strictly anaerobic bacteria (e.g., clostridia), and the facultatively or strictly aerobic bacteria (e.g., *Staphylococcus* and *Bacillus*) (Axelsson, 1993).

The lactic acid bacteria are a heterogeneous group of non-sporeforming, catalase-negative, coccus, coccobacilli or rod-shaped microorganisms, which by definition produce lactic acid as the principal end product of fermentation. The metabolism of LAB is either homofermentative or heterofermentative. Homofermentative LAB produce mainly lactate as the major fermentation end product, whereas heterofermentative LAB produce additional products such as acetate, ethanol and carbon dioxide.

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* A version of this chapter entitled **Enterococci at the crossroads of food safety?** has been submitted for publication to the International Journal of Food Microbiology by Charles M.A.P. Franz, Wilhelm H. Holzapfel and Michael E. Stiles.

The taxonomy of lactic acid bacteria is based on morphological and physiological features as well as the more modern molecular tools such as mol% G+C content of the DNA, electrophoretic properties of gene products, DNA:DNA hybridization studies and structures and sequence of ribosomal RNA (rRNA) (Stiles and Holzapfel, 1997). The LAB group currently includes the following genera: *Aerococcus, Alloiococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Schleifer and Ludwig, 1995; Stiles and Holzapfel, 1997). Despite great advances in LAB taxonomy over the past years the classification of LAB is in a state of constant flux and it is the focus of intense study (Stiles and Holzapfel, 1997). The need for a polyphasic approach of modern taxonomy involving phenotypic, genotypic and phylogenetic information is recognized and applied in LAB taxonomy (Vandamme et al., 1996).

1.1.2. *Lactic acid bacteria as food spoilage organisms.*

Lactic acid bacteria are well known for their preservative effect in food fermentations; however, their role in spoilage of foods is also well documented. For example, lactic acid bacteria are well adapted for growth in refrigerated, modified atmosphere or vacuum-packaged cooked meats and poultry (Huis in't Veld, 1996). The combination of microaerophilic conditions in packages, the presence of curing salts and nitrite and low storage temperatures favors the growth of resistant LAB and they usually represent the predominant spoilage population of these products (Borch et al., 1996; Huis in't Veld, 1996). Lactic acid bacteria spoil packaged meats by fermentation of sugars to form organic acids, carbon dioxide and slime, which lead to a drop in pH and off-flavors (Huis in't Veld, 1996). The main LAB genera associated with spoilage of packaged meats are *Lactobacillus* and *Leuconostoc* (Borch et al., 1996). Sulfide-producing *L. sake* strains have been associated with sulfide-spoilage of vacuum-packaged meat (Egan et al., 1989). Spoilage of unprocessed, fresh meat products by LAB can also occur, especially when such products are vacuum-packaged or modified atmosphere packaged. The role of LAB in spoilage of fresh meats and the use of bacteriocin-producing LAB in preservation

of meats is actively being investigated in our research group and will be discussed in more detail below.

Other examples of LAB in food spoilage include: cracking defects due to gas production in Gouda or Edam cheese caused by *L. bifermentans;* spoilage of citrus fruits, wines and beer by *L. brevis;* spoilage of beer by *Pediococcus damnosus;* 'greening' of cured meat products by *Weissella viridescens* (Hammes et al., 1991); and spoilage in sugar processing as a result of production of dextrans by leuconostocs (Stiles and Holzapfel, 1997).

1.1.3. *Lactic acid bacteria in food preservation.*

The lactic acid bacteria have been used for food preservation, knowingly or unknowingly, since ancient times. For example, excavations in Switzerland have shown that sourdough bread, a LAB fermented product, was part of a typical diet over 5000 years ago (Währen, 1990). Fermented dairy products are mentioned in archaic texts from Uruk/Warka (today's Iraq) dated around 3200 BC (Nissen et al., 1991). Pasteur contributed significantly to research on lactic acid fermentation in 1857, and the use of lactic starter cultures for cheese and sour milk production was introduced in 1890 in both Germany and Denmark (Stiles and Holzapfel, 1997).

Lactic acid bacteria are involved in fermentations of dairy, vegetable, milk and cereal products (Lindgren and Dobrogosz, 1990; Hammes and Tichaczek, 1994; Stiles and Holzapfel, 1997). Historically, some of these food fermentations were based on empirical processes involving the natural microflora present on the raw material combined with technical manipulations (e.g., mincing, chopping or tight packaging to enhance distribution of fermentative flora and the systems anaerobicity), or additions (e.g., addition of salt, sugar or 'back slopping' of starter material) (Lindgren and Dobrogosz, 1990). It was estimated that 25% of the European diet and 60% of the diet in developing countries consists of fermented foods (Holzapfel et al., 1995). Lactic fermentations improve food flavor, aroma and texture (Lindgren and Dobrogosz, 1990; Holzapfel et al., 1995; Stiles, 1996).

The preservative action of LAB in foods is a result of formation of fermentation end-products with antimicrobial activity (Lindgren and Dobrogosz, 1990; Holzapfel et al., 1995). Such antimicrobial compounds include: organic acids (e.g., lactic, acetic and formic); carbon dioxide; hydrogen peroxide (in the presence of oxygen); diacetyl; aldehydes (e.g., β-hydroxypropionaldehyde) and bacteriocins (Lindgren and Dobrogosz, 1990; Gould, 1992; Hammes and Tichaczek, 1994; Holzapfel et al., 1995).

Bacteriocins have received great interest in recent years for potential application as food 'biopreservatives'. Consumers today demand food of high quality, which is less severely processed (less intensive heating and minimal freezing damage), less heavily preserved, more natural (free of artificial additives) and safer (Ohlsson, 1994; Gould, 1992, 1996). These demands are addressed in the marketplace by the emergence of a new generation of chill stored, minimally-processed foods (Ohlsson, 1994; Stiles, 1996). These foods rely on processing procedures that change the inherent fresh-like quality attributes of the food as little as possible (minimally) but at the same time endow the food with a sufficient shelf life (Ohlsson, 1994). However, minimal processing may lead to a loss of intrinsic preservation (e.g., less sugar, salt, preservatives) and to a loss in protection from processing (e.g., less severely heated) (Gould, 1992). As a result, new preservation technologies are sought that employ 'natural' antimicrobial agents for preservation, and that prolong shelf life and safeguard food from foodborne pathogens, but do not have a detrimental effect on quality attributes of the food (Ohlsson, 1994; Holzapfel et al., 1995). Such a 'natural' preservation possibility is offered by the 'biopreservation' technology which makes use of either a 'natural' LAB microflora as so-called 'protective culture', or their antimicrobial metabolites, notably bacteriocins (Holzapfel et al., 1995; Stiles, 1996). Many LAB are considered to be 'food grade' organisms, because they have been involved in numerous food fermentations known to man for millennia, and some are designated as GRAS (generally recognized as safe) organisms (Holzapfel et al., 1995). With the exception of some streptococci, LAB are rarely pathogenic to humans and animals (Aguirre and Collins, 1993; Gasser, 1994). However, LAB can act as opportunistic pathogens, especially in immunosuppressed patients or patients with underlying disease (Aguirre and Collins, 1993). Generally these

are rare cases, but a notable exception is the involvement of *Enterococcus* spp. in human infections, and this will be discussed in more detail below.

1.2. Bacteriocins produced by lactic acid bacteria.

1.2.1. *Definition and classification of bacteriocins.*

Bacteriocins are produced by both gram-negative and gram-positive bacteria and it was bacteriocin production by the gram-negative bacteria that received initial attention with the discovery of an antimicrobial agent produced by *E. coli* strain V (virulent in experimental infections) that was later referred to as colicin V (Gratia, 1925; Jack et al., 1995). This was followed by a period of discovery of new colicins produced by *E. coli* and closely related members of the *Enterobacteriaceae*. It soon became clear that the unusually small size and heat stability of colicin V set it apart from the colicins that were subsequently isolated. Colicin V has a double-glycine-type leader peptide (Håvarstein et al., 1994) and it does not appear to be posttranslationally modified; therefore, it more appropriately fits the description of a class II bacteriocin (see below).

Discovery of the production of antimicrobial substances by gram-positive bacteria followed, with the term 'bacteriocin' coined by Jacob et al. (1953). A review by Tagg et al. in 1976 on bacteriocins produced by gram-positive bacteria defined these compounds as 'proteinaceous compounds that kill closely related bacteria'. Bacteriocin production has been described for all genera of LAB (DeVuyst and Vandamme, 1994). In a review on the genetics of bacteriocins produced by LAB, Klaenhammer (1993) recognized that while the definition of Tagg et al. (1976) held true in many cases, it was evident that bacteriocins could 'take many forms and elicit bactericidal activity beyond species that are closely related'. Klaenhammer (1993) also took into account reports that surfaced at that time, that claimed that bacteriocins could be composed of protein with additional carbohydrate or lipid moieties.

Four distinct classes of LAB bacteriocins were defined by Klaenhammer (1993): Class I bacteriocins are also known as the 'lantibiotics' and these are small, ribosomally synthesized peptides that undergo extensive posttranslational modification. They contain

unusual amino acids such as lanthionine and β-methyllanthionine, and dehydrated residues. The best characterized lantibiotic is nisin. It is used world-wide in preservation of foods such as processed cheeses and canned fruits and vegetables (Hurst, 1981; Delves-Broughton, 1990; Holzapfel et al., 1995). Class II bacteriocins are small, heat stable peptides that do not undergo extensive posttranslational modification except for cleavage of a leader peptide during transport, and in some cases the formation of disulfide bridges between cysteine residues. These bacteriocins are produced as prepeptides that contain a 'double-glycine-type' leader peptide, which is characterized by a conserved processing site of two glycines at positions –2 and –1. Three subgroups of class II bacteriocins were defined: Class IIa are the '*Listeria*-active' peptides which contain a conserved N-terminal YGNGVXC-consensus sequence; class IIb are poration complexes consisting of two proteinaceous peptides for activity; and class IIc are thiol-activated peptides requiring reduced cysteine residues for activity. Class III bacteriocins are large, heat labile proteins while class IV bacteriocins are complex bacteriocins composed of protein as well as additional chemical moieties such as lipid or carbohydrate (Klaenhammer, 1993). The class IV bacteriocins are the least characterized and direct evidence for a requirement of an additional chemical moiety for activity is lacking.

Nes et al. (1996) re-grouped the class II bacteriocins: Class IIa bacteriocins are the 'pediocin-like' bacteriocins which contain the YGNGVXC-consensus motif; class IIb are the two-peptide bacteriocins; and class IIc are bacteriocins that are secreted by the *sec*-pathway and which contain a typical signal peptide that is required for *sec*-dependent secretion. While Nes et al. (1996) did not re-classify class I and III bacteriocins as defined by Klaenhammer (1993), they excluded class IV bacteriocins because they are not yet adequately characterized at the chemical level. Also, they suggested that the observed complex bacteriocinogenic activities could be artifacts, caused by interaction of protein with cell or medium constituents.

While these classifications of bacteriocins allow grouping of many bacteriocins into one of the respective classes, some bacteriocins display unusual characteristics that do not allow assignment to any of the existing bacteriocin classes. For example the bacteriocins, carnobacteriocin A (Worobo et al., 1994) and enterocin B (this study) do not contain the YGNGVXC-consensus motif at the N-terminus of the respective peptides.

Although these bacteriocins are *Listeria*-active, similar to other class IIa bacteriocins, they clearly lack the 'pediocin-like' structure. The bacteriocin AS-48 is a cyclic molecule (Martínez-Bueno et al., 1994) while the bacteriocin/hemolysin produced by many *E. faecalis* strains is a two-component lantibiotic (Booth et al., 1996). Enterocins L50A and L50B are novel in that they do not require either a leader peptide or signal peptide for secretion (Cintas et al., 1998). Hence, the current classification schemes of Klaenhammer (1993) and Nes et al. (1996) are inadequate. However, Nes et al. (1996) noted that 'subgrouping of bacteriocins is a way to organize the present knowledge in a functional way'; because the knowledge of bacteriocins is increasing, and novel types of bacteriocins have been recently discovered that do not fit the current classification scheme, bacteriocin classification may have to be revised again in the future.

1.2.2. *Genetics of bacteriocin production.*

In the 1990's there has been an 'explosion' of research activity on the bacteriocins produced by LAB (Klaenhammer, 1993). The majority of this research appears to be concerned with class II bacteriocins, although the genetics of production of the important food preservative nisin has also received considerable attention. Research in our group is mainly concerned with class II bacteriocins. Because the genetics of the class II bacteriocin enterocin B is a central component of this study, only the genetics of bacteriocin production for class II bacteriocins will be discussed here.

The genetic determinants for expression of most well characterized class II bacteriocins are organized in operons consisting of at least four genes: the bacteriocin structural gene encoding the prebacteriocin, a dedicated immunity gene generally located next to the bacteriocin gene and on the same transcription unit, a gene encoding a dedicated ATP-binding casette (ABC) transporter which externalizes the bacteriocin concomitant with cleavage of the leader peptide, and an accessory gene which encodes a protein for which the specific role is not known but it appears to be essential for transport of the bacteriocin out of the cell (Klaenhammer et al., 1993; Nes et al., 1996). These four genes are organized either in one or two operons (Nes et al., 1996). In the case of class IIc bacteriocins the genes for dedicated-bacteriocin secretion are missing because these

bacteriocins are externalized by proteins of the bacterial preprotein translocase (den Blaauwen and Driessen, 1996; Economou, 1998), which are also known as the general secretory (*sec*) pathway proteins (Pugsley, 1993; Economou, 1998). In addition to the four 'basic' genes, bacteriocin operons may also contain regulatory genes necessary for bacteriocin production.

1.2.2.1. *The bacteriocin and its gene*. The structural bacteriocin gene encodes a prebacteriocin (prepeptide) containing an N-terminal leader sequence (Fig. 1.1). The N-terminal leader sequence renders the bacteriocin molecule biologically inactive, and provides a recognition signal for the transport system (Nes et al., 1996; van Belkum et al., 1997).

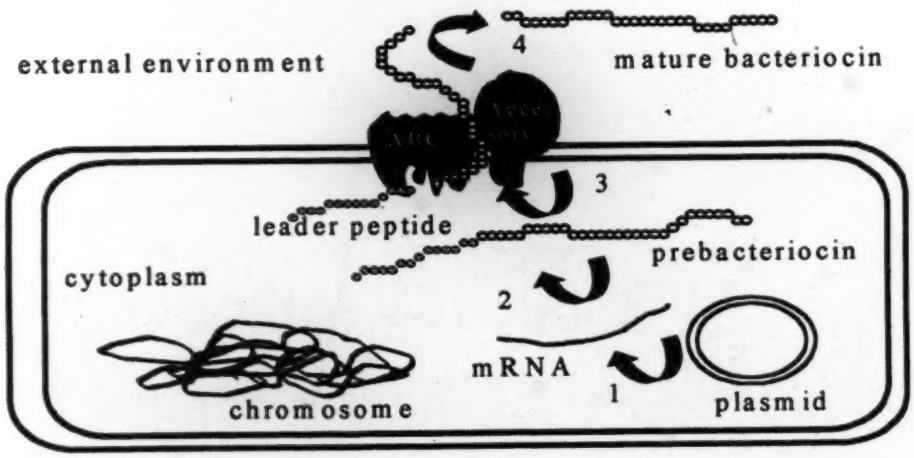

Figure 1.1 Schematic representation of bacteriocin secretion by the dedicated-secretion pathway. Plasmid DNA in the cell encoding the bacteriocin structural gene is transcribed to messenger RNA (mRNA) (1), which is subsequently translated to form the prebacteriocin (2). The prebacteriocin contains a double-glycine-type leader peptide which is cleaved (3) by the ABC transporter (ABC) concomitant with export. Secretion is also dependent on the presence of the accessory protein (accessory), and mature bacteriocin is released into the external environment (4). Figure based on reviews from Klaenhammer (1993) and Nes et al. (1996).

These leader peptides are commonly cleaved following a conserved glycine (-2) glycine (-1) processing site, and for this reason they are referred to as 'double-glycine-type' leader peptides (Håvarstein et al., 1995; Nes et al., 1996). Double-glycine-type leader peptides vary in length from 14 to 30 amino acids (Klaenhammer, 1993; Håvarstein et al., 1994). They are removed from the prebacteriocin to yield mature bacteriocin by the ABC-transporter protein concomitant with export (Håvarstein et al., 1995) (Fig. 1.1). The following consensus sequence was determined for double-glycine-type leader peptides: L(-12), S(-11), X(-10), X(-9), E(-8), L(-7), X(-6), X(-5), I(-4), X(-3), G(-2) and G(-1), and the glycine at the –2 position is 100% conserved in different double-glycine-type leaders (Håvarstein et al., 1994; Nes et al., 1996) (Table 1.1). Examples of double-glycine-type leader peptides of class II bacteriocins are shown in Table 1.1

Bacteriocins that are transported by the bacterial preprotein translocase do not possess double-glycine-type leaders but bear signal peptides. These signal peptides are recognized and cleaved by the signal peptidase of the bacterial preprotein translocase concomitant with transport (discussed below). Signal peptides share the following common features: they have a positively charged N-terminal domain of two to fifteen residues followed by a hydrophobic domain composed of more than eight, predominantly hydrophobic residues (Pugsley, 1993). The C-terminal domain is less hydrophobic and contains the signals recognized by the signal peptidase, which are neutral amino acids with small side chains (e.g., Ala, Gly, Ser) at positions –1 and –3 relative to the processing site, and a turn inducing residue (usually Pro or Gly) may be present at position –6 (Von Heijne, 1983; Pugsley, 1993). Signal peptides associated with bacteriocin secretion are shown in Table 1.2.

Mature class II bacteriocins vary in size from 30 to more than 100 residues (Nes et al., 1996). These bacteriocins share a number of common features: they have a high content of small amino acids, e.g., glycine; they are strongly cationic; their pI's generally vary from 8 to 11; and they possess a hydrophobic domain and/or amphiphilic region which may relate to their activity on membranes (Abee, 1995; Nes et al., 1996). A number of bacteriocins are known which consist of two peptides (class IIb), both of which possess a double-glycine-type leader.

Table 1.1 Double-glycine-type leader peptides of class II bacteriocins.

Class II bacteriocin	Double-glycine-type leader peptide sequence	Reference
Brochocin A[a]	MHKVKKLNNQELQQIVGG Y..	McCormick et al., 1998
Brochocin B[b]	MKKELLNKNEMSRIIGG K..	McCormick et al., 1998
Carnobacteriocin A	MNNVKELSIKEMQQVTGG D..	Worobo et al., 1994
Carnobacteriocin B2	MNSVKELNVKEMKQLHGG V..	Quadri et al., 1994
Carnobacteriocin BM1	MKSVKELNKKEMQQINGG A..	Quadri et al., 1994
Carnobacteriocin X	MKSVKELNVKEMQQTIGG W..	Quadri et al., 1997
Carnobacteriocin Y	MNKEFKSLNEVEMKKINGG S..	Quadri et al., 1997
Enterocin A	MKHLKILSIKETQLIYGG T..	Aymerich et al., 1996
Lactacin A[a]	MKQFNYLSHKDLAVVVGG R..	Fremaux et al., 1993
Lactacin X[a]	MKLNDKELSKIVGG N..	Fremaux et al., 1993
Lactococcin A	MKNQLNFNIVSDEELSEANGG K..	Holo et al., 1991
Lactococcin B	MKNQLNFNIVSDEELAEVNGG S..	van Belkum et al., 1991
Lactococcin Gα[a]	MKELSEKELRECVGG G..	Håvarstein et al., 1995
Lactococcin Gβ[a]	MKNNNNFFKGMEIIEDQELVSITGG K..	Håvarstein et al., 1995
Lactococcin M[a]	MKNQLNFEILSDEELQGINGG I..	van Belkum et al., 1991
Lactococcin N[a]	MKKDEANTFKEYSSSFAIVTDEELENINGS G..	van Belkum et al., 1991
Leucocin A	MMNMKPTESYEQLDNSALEQVVGG K..	Hastings et al., 1991
Leucocin B	MNNMKSADNYQQLDNNALEQVVGG K..	Hastings et al., 1994
Mesentericin Y105	MTNMKSVEAYQQLDNQNLKKVVGG K..	Héchard et al., 1993
Sakacin A	MNNVKELSMTELQTITGG A..	Axelsson and Holck, 1995
Sakacin P	MEKFIELSLKEVTAITGG K..	Tichaczek et al., 1994
Pediocin PA-1	MKKIEKLTEKEMANIIGG K..	Marugg et al., 1992
Plantaricin E	MMLQFEKLQYSRLPQKKLAKISGG F..	Diep et al., 1996
Plantaricin F	MKKFLVLRDRELNAISGG V..	Diep et al., 1996
Plantaricin J	MTVNKMIKDLDVVDAFAPISNNKLNGVVGG G..	Diep et al., 1996
Plantaricin K	MKIKLTVLNEFEELTADAEKNISGG R..	Diep et al., 1996
Consensus[b]	LS EL I GG X..	Håvarstein et al., 1994
	# #* **# #	

[a] two-component bacteriocin

[b] #, hydrophobic residue; *, hydrophilic residue

Examples of such two-component bacteriocins with leader peptides include lactococcin G (Nissen-Meyer et al., 1992), lactacin F (Allison et al., 1994) and brochocin C (McCormick et al., 1998).

A recently described two-component bacteriocin consists of the enterocin L50A and L50B peptides, both of which do not possess either a leader or a signal peptide (Cintas et al., 1998). Expression of these peptides apparently does not rely on either the bacteriocin-dedicated secretion machinery or the bacterial preprotein translocase (Cintas et al., 1998).

Table 1.2 Signal peptides associated with class II bacteriocins.

Class IIc bacteriocin	Signal peptide sequence	Reference
Acidocin B	MVTKYGRNLGLSKVELFAIWAVLVVALLLATA N..	Leer et al., 1995
Bacteriocin 31	MKKKLVICGIIGIGFTALGTNVEAAT Y..	Tomita et al., 1996
Enterocin P	MRKKLFSLALIGIFGLVVTNFGTKVDA A..	Cintas et al., 1997
Divergicin A	MKKQILKGLVIVVCLSGATFFSTPQASA A..	Worobo et al., 1995

1.2.2.2. *The immunity protein and its gene.* The immunity protein confers protection to the producer strain from its own bacteriocin (Jack et al., 1995; Nes et al., 1996). Each bacteriocin has its own dedicated protein conferring immunity and generally this is expressed concomitantly with the bacteriocin because the bacteriocin structural and immunity genes occur in an operon (Klaenhammer, 1993; Jack et al., 1995; Nes et al., 1996). Bacteria that produce more than one bacteriocin produce corresponding, specific immunity proteins that are responsible for self protection to each bacteriocin (Jack et al., 1995). For example *L. lactis* subsp. *cremoris* 9B4 produces three different bacteriocins, lactococcins A, B and M, as well as three corresponding immunity proteins (van Belkum et al., 1991, 1992). While bacteriocin production relies on a dedicated secretion/processing system, the immunity protein is functionally expressed in the absence of transport or processing (Nes et al., 1996). Immunity proteins are relatively small, ranging from 51 to 257 amino acids (Table 1.3) and homology between various immunity proteins is surprisingly low when considering the similarity between different

Table 1.3 Characteristics of immunity genes of class II bacteriocins.

		Immunity protein characteristics		
Bacteriocin system	**Immunity gene**	**Size (amino acids)**	**pI**	**Reference**
Carnobacteriocin B2	*cbiB2*	111	9.3	Quadri et al., 1994, 1995
Curvacin A		51		Tichaczek et al., 1993
Divergicin A	*dviA*	56	10.3	Worobo et al., 1995
Enterocin A	*orf2*	103		Aymerich et al., 1996
Enterocin P	*orf2*	88		Cintas et al., 1997
Lactacin F	*lafI*	124	9.8	Allison and Klaenhammer, 1996
Lactococcin A	*lciA*	98	10.2	Nissen-Meyer et al., 1993a; Venema et al., 1994
Lactococcin B	*lciB*	91	9.8	Van Belkum et al., 1992
Lactococcin M/N	*LciM*	154	10.1	Van Belkum et al., 1991
Leucocin A	*lcaB*	113	9.5	Van Belkum and Stiles, 1995
Pediocin PA-1	*pedB*	122	7.4	Venema et al., 1995
Plantaricin E/F	*plnI*	257	9.7	Diep et al., 1996
Plantaricin J/K	*plnL*	138	7.3	Diep et al., 1996
Sakacin A	*saiA*	90	10.1	Axelsson and Holck, 1995
Sakacin P	*spiA*	98		Hühne et al., 1996; Brurberg et al., 1997

bacteriocins (Nes et al., 1996). For example, the immunity proteins of the identical bacteriocins sakacin A and curvacin A are 90 and 51 amino acids, respectively (Axelsson et al., 1993; Tichaczek et al., 1993). Immunity proteins are cationic and their pI's typically range from 9 to 10 (Allison and Klaenhammer, 1996) (Table 1.3).

The immunity protein of lactococcin A (LciA) has been purified and it was shown that LciA is present in approximately equal amounts in the cytoplasm and membrane fractions (Nissen-Meyer et al., 1993; Venema et al., 1994). LciA was suggested to have an amphiphilic α-helix domain (residue 9 to 47) which may serve to anchor LciA to the cytoplasmic membrane. Because membrane vesicles obtained from an immune strain are resistant to lactococcin A, it was concluded that LciA is located in and acts at the level of the cytoplasmic membrane. Free intracellular LciA is considered a reservoir of immunity protein for use when needed.

In a similar study, Quadri et al. (1995) purified the immunity protein for carnobacteriocin B2 (CbiB2) and determined that the majority (92%) of the intracellular pool of this protein was in the cytoplasm, while the remaining smaller proportion (8%) was associated with a membrane fraction. The purified immunity protein did not interact with the carnobacteriocin B2 molecule (CbnB2), i.e., it did not bind to microtiter plates coated with CbnB2 and it did not inactivate the bacteriocin in solution (Quadri et al., 1995). In contrast to LciA, no transmembrane helix could be predicted for the CbiB2 molecule. Because CbnB2 targets the cytoplasmic membrane of sensitive cells and probably causes pore formation (Quadri et al., 1995), it was suggested that CbiB2 may block the pore from the cytoplasmic side or prevent pore formation (Quadri et al., 1995). On the other hand, the proteins LafI and LciM conferring immunity to the bacteriocins lactacin F and lactococcin M (Allison and Klaenhammer, 1996; van Belkum et al., 1991), respectively, were predicted to contain four transmembrane helices (Allison and Klaenhammer, 1996). Also, the lactococcin A immunity protein LciA was shown to have an α-helical domain that could associate with the membrane (Venema et al., 1994). Because LafI, LciM and LciA could associate with the cytoplasmic membrane, it was suggested that these immunity proteins interact with a receptor molecule in the cytoplasmic membrane and prevent bacteriocin binding (Venema et al., 1994; Allison

and Klaenhammer, 1996). However, the presence of such receptors is speculative and such molecules have yet to be identified (Venema et al., 1994; Allison and Klaenhamer, 1996).

1.2.2.3. *Dedicated bacteriocin transport: ABC transporter and accessory protein genes.* As mentioned above, bacteriocins that are secreted by the dedicated bacteriocin transport system contain a leader peptide of the double-glycine type. Double–glycine-type leader peptides are recognized by transmembrane translocators belonging to the ATP-binding Cassette (ABC) transporter superfamily. Members of the ABC transporter superfamily transport a wide range of substrates (e.g., inorganic ions, sugars, amino acids, peptides, proteins) across the cytoplasmic membrane in prokaryotes and eukaryotes (Higgins et al., 1992). The bacteriocin ABC-transporter gene is usually part of the bacteriocin operon, or it is found in a separate operon in the vicinity of the bacteriocin-containing operon (Håvarstein et al., 1995; Nes et al., 1996). ABC transporters consist of two protein domains: a C-terminal ATP-binding domain of about 200 amino acids which is located in the cytoplasm, and a N-terminal hydrophobic integral membrane domain (normally consisting of six membrane spanning segments). The individual domains may be expressed as separate polypeptides or together as a single polypeptide chain (Hyde et al., 1990). Two such polypeptides, i.e., two N-terminal and two C-terminal domains, are required to make a functional ABC transporter (Higgins, 1992). The energy needed for translocation is provided by the hydrolysis of ATP (Håvarstein et al., 1995).

Håvarstein et al. (1995) showed that the ABC transporters of bacteriocins (e.g., LcnC of lactococcins A, B and M; PedD of pediocin PA-1; and CvaB of colicin V) contain an additional N-terminal domain, which is located in the cytoplasm. This domain is absent in other ABC transporters, for example the ABC transporter for the *E. coli* α-hemolysin (HlyB). In ABC transporters for bacteriocins this domain functions as a protease and removes the double-glycine type-leader peptide concomitant with secretion (Fig. 1.1). Therefore, ABC transporters associated with dedicated bacteriocin secretion contain a cytoplasmic, N-terminal proteolytic domain, six helical membrane spanning segments, and a cytoplasmic C-terminal domain (Fig. 1.2). It has been shown that mutations affecting the conserved glycine residue at position –2 (see above) in leader

peptides of lactacin F and colicin V result in loss of bacteriocin secretion (Gilson et al., 1990; Fremaux et al., 1993). This suggested that the conserved glycine residue is part of the consensus cleavage site of the leader protease and functions as an export signal for bacteriocin secretion (Håvarstein et al., 1995). Van Belkum et al. (1997) investigated the role of leader peptides in bacteriocin secretion by fusing the leader peptides of leucocin A, lactococcin A and colicin V to divergicin A, a bacteriocin from *Carnobacterium divergens* that is normally secreted by the bacterial preprotein translocase (Worobo et al., 1995). The different leader peptides were shown to direct divergicin secretion in homologous and heterologous hosts, showing that they function as transport signals (van Belkum et al., 1997).

In addition to the gene for an ABC transporter, dedicated bacteriocin transport relies on the presence of a gene for an accessory protein. The products of accessory genes consist of approximately 470 amino acids (Nes et al., 1996). Computer assisted amino acid sequence analysis of the lactococcin A accessory protein (LcnD) predicted an N-terminal transmembrane sequence (Stoddard et al., 1992). The localization of the transmembrane structure of LcnD was confirmed in a topological study using various in-frame translation fusions to reporter proteins (Franke et al., 1996). The study of Franke et al. (1996) showed that the N-terminus of LcnD is intracellularly located, while the transmembrane segment (residues 22 to 43) is followed by an externally located C-terminal domain of 431 amino acids (Fig. 1.2). Based on computer predictions, accessory proteins associated with secretion of other bacteriocins have similar topology characteristics (Franke et al., 1996). The specific role of the accessory protein in the translocation process has not yet been elucidated (Nes et al., 1996).

1.2.2.4. *Bacteriocin transport by the bacterial preprotein translocase.* Bacteriocins which are transported by the bacterial preprotein translocase bear signal peptides. The signal peptide is recognized by the translocase and acts as a targeting signal that guides preproteins to the translocase at the cytoplasmic membrane (den Blaauwen and Driessen, 1996). The following discussion on preprotein secretion by translocase subunit proteins applies also to secretion of bacteriocins. Heterologous expression of bacteriocin by this pathway is an important part of this study and the preprotein translocase will therefore be

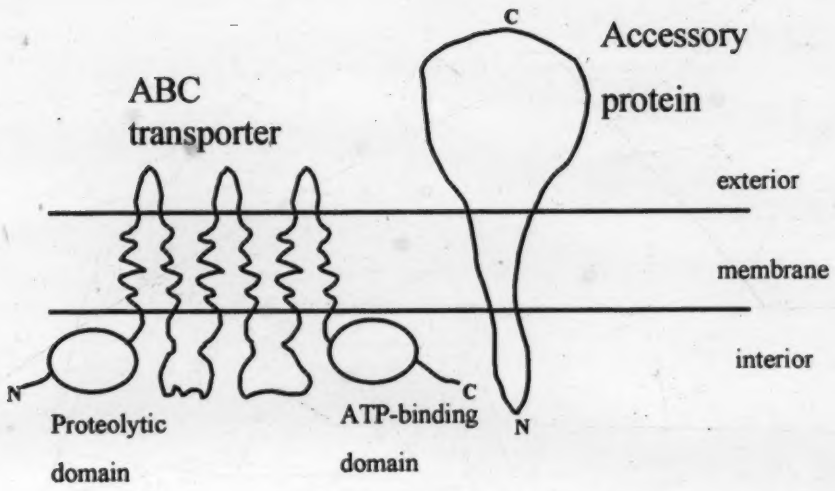

Figure 1.2 Diagram showing the organization of domains of ABC transporter and accessory proteins. These domains include a N-terminal proteolytic domain and a C-terminal ATP-binding domain separated by six membrane spanning helices for the ABC transporter, and a relatively small cytoplasmic N-terminal domain and large, external C-terminal domain separated by a membrane spanning segment for the accessory protein. Figure adapted from Nes et al. (1996) and is based on data from Franke et al. (1996).

discussed in more detail. Targeting of preproteins to the membrane is generally facilitated by a cytosolic chaperone protein such as SecB (Kumamoto, 1991; Randall and Hardy, 1995). SecB binds to the mature region of the preprotein and prevents native folding or aggregation (Pugsley, 1993; Economou, 1998). The translocase is a multi-subunit protein complex which consists of the integral membrane subunits SecY, SecE, SecG, SecD and SecF and the dissociable peripheral subunit SecA (den Blaauwen and Driessen, 1996). These subunits have been found in all prokaryotes studied thus far (den Blaauwen and Driessen, 1996). The chaperone SecB has a high affinity for the membrane associated translocase and, in particular, for the SecA subunit, which in turn promotes the

interaction of the preprotein with SecA (den Blaauwen and Driessen, 1996; Economou, 1998). SecA functions as a dimer and can bind and hydrolyze ATP at two nucleotide binding sites (den Blaauwen and Driessen, 1996; Economou, 1998). It is a cytosolic protein but can also partially insert into the cytoplasmic membrane. SecA interacts with the preprotein through recognition of the positive charge at the N-terminus and by binding the mature domain of the preprotein. SecA interacts at the membrane with SecYEG protein and this binding stimulates ATP hydrolysis and membrane insertion (Economou, 1998). Membrane insertion displaces SecB from SecA and initiates translocation (den Blaauwen and Driessen, 1996).

The integral membrane subunits of the translocase SecY, SecE and SecG assemble *in vivo* as a stable heterotrimeric complex, SecYEG. Membrane insertion of SecA makes the signal peptide domain of the bound preprotein accessible to SecY and SecE. When these proteins recognize and bind to the signal sequence, the translocase channel opens and allows access of the N-terminal domain of SecA with the mature part of the preprotein (den Blaauwen and Driessen, 1996). This stimulates the ATPase activity of SecA causing it to release the preprotein and to withdraw from the membrane (den Blaauwen and Driessen, 1996; Economou, 1998). The other integral membrane proteins SecD and SecF are important for efficient protein secretion and are involved in the late stage of translocation. SecD and SecF are believed to be 'translocation release factors' involved with releasing the preprotein from the translocation channel (Pugsley, 1993). Signal peptide cleavage by the signal peptidase is a last step in translocation in gram-positive bacteria. The SecY gene of *L. lactis* has been cloned and sequenced (Koivula et al., 1991), indicating that protein secretion by the bacterial preprotein translocase occurs in LAB and is homologous to other bacteria. A schematic representation of secretion of LAB bacteriocins by the bacterial preprotein translocase in accordance with the above discussion is shown in Fig. 1.3.

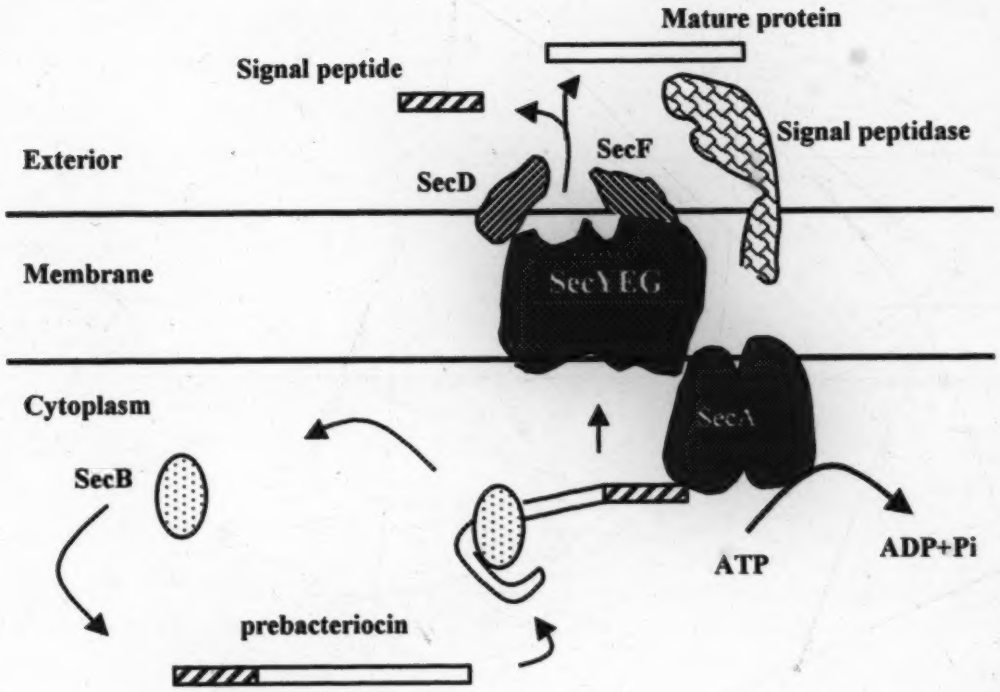

Figure 1.3 Diagram showing transport of class IIc bacteriocins bearing signal peptides by the bacterial preprotein translocase (see text for details). Figure assimilated according to data from Pugsley (1993) and Economou (1996).

1.2.3. *Regulation of class II bacteriocin production.*

Several gene expression mechanisms in gram-positive bacteria are regulated in a cell-density-dependent manner. Examples of such 'quorum-sensing' systems are the development of genetic competence in *Streptococcus pneumoniae*, the virulence response in *S. aureus* and the production of bacteriocins in several different species of LAB (Kleerebezem et al., 1997). Cell-density-dependent regulation in these systems follows a common theme, in which the signal molecule, also known as induction factor (IF) or

pheromone, is a posttranslationally processed peptide, that is secreted by dedicated ABC transporter and accessory proteins. This secreted peptide functions as the input signal for a specific sensor component of a two component signal transduction system consisting of a histidine protein kinase and a response regulator (Stock et al., 1989; Nes et al., 1996; Kleerebezem et al., 1997) (Fig. 1.4). The secreted induction factor is sensed by the sensor component of the histidine kinase at the cell exterior. A C-terminal transmitter located in the cytoplasm has autokinase activity and a conserved histidine residue as the site for phosphorylation. The response regulator also consists of two components, a N-terminal receiver containing a conserved aspartic acid residue as the site for phosphorylation, and a C-terminal output domain. Communication between the histidine kinase and response regulator thus involves phosphorylation and dephosphorylation, which leads to regulation of gene expression (Nes et al., 1996; Kleerebezem et al., 1997) (Fig. 1.4). A common feature in many of the quorum sensing models is that the genes for the induction factor preprotein and the genes encoding the two component signal transduction system are transcriptionally linked and that the synthesis of the induction factor is autoregulated. Therefore, both induction factor production and the cell-density-dependent phenotype (bacteriocin production) are regulated by the response regulator (Kleerebezem et al., 1997) (Fig. 1.4).

It is well established that bacteriocin production can be a cell-density-dependent phenotype. Production of carnobacteriocins A, B2 and BM1 by *C. piscicola* LV17 (Saucier et al., 1995; Quadri et al., 1994,1997), putative plantaricins (PlnJK, PlnEF and PlnN) by *L. plantarum* C11 (Diep et al., 1995, 1996), and sakacin P by *L. sake* LTH673 (Eijsink et al., 1996) was lost upon inoculation of an overnight culture of a producing (Bac^+) strain into fresh culture medium at a level lower than a certain threshold inoculum size (varying from $<10^6$ to $<10^4$ CFU/ml). The Bac^- phenotype persisted during subsequent subcultivation in an inoculum-size-independent manner, but could be reversed by addition of a small volume (from 0.01 to 1% v/v) of cell free, Bac^+ culture supernatant (Kleerebezem et al.,1997).

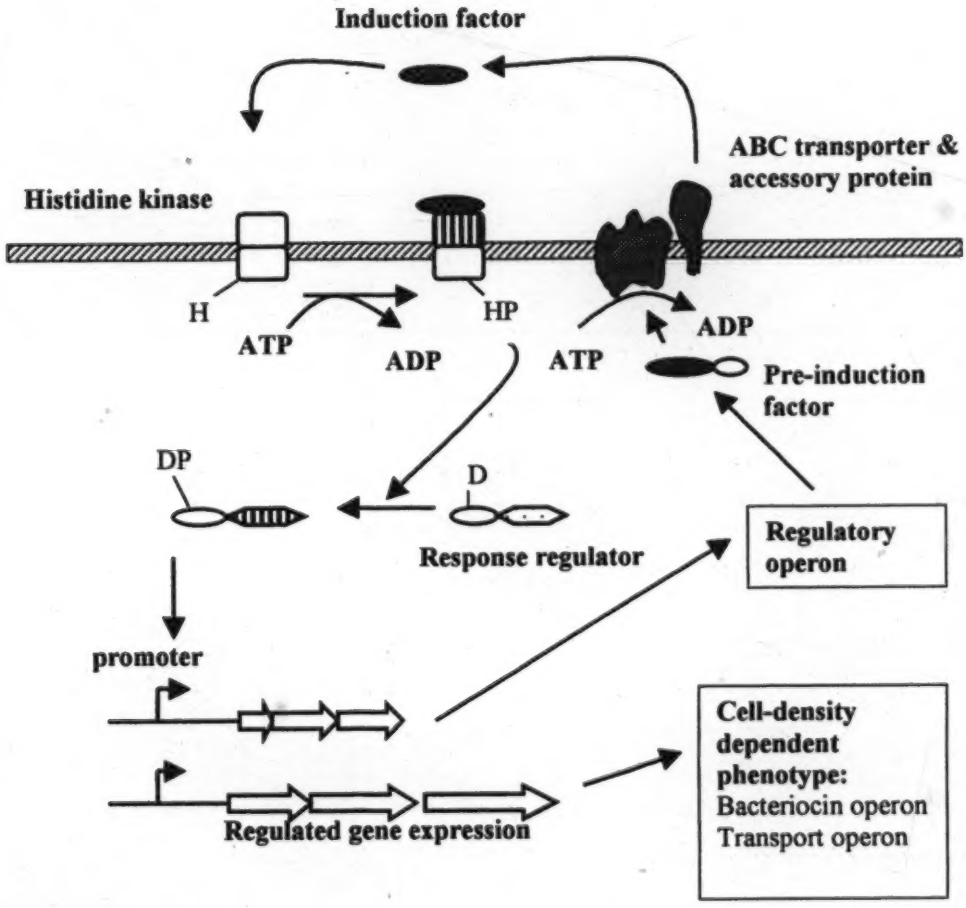

Figure 1.4 Schematic representation of a model for induction of bacteriocin production mediated by the induction factor and two-component regulatory system (H=histidine residue; D= aspartic acid residue, P=phosphorylated residue, see text for detail). Figure adapted from Kleerebezem et al. (1997).

Nucleotide sequencing of class II bacteriocin operons has shown that regulatory genes are associated with production of several putative plantaricins (PlnJK, PlnEF and PlnN) by *L. plantarum* C11 (Diep et al., 1995, 1996), sakacin P production by *L. sake* LTH673 and Lb674 (Huehne et al., 1996; Brurberg et al., 1997), carnobacteriocin A and B2 production by *C. piscicola* LV17 (Worobo, 1996; Quadri et al., 1997), enterocin A and B production by *Enterococcus faecium* CTC492 (Nilsen et al., 1998) and sakacin A production by *L. sake* Lb706 (Axelsson and Holck, 1995). Genes for induction factors have been described for most of these two component regulatory systems. The induction factor gene, histidine protein kinase and response regulator genes together make up a three-component regulatory system (Nes et al., 1996; Kleerebezem et al., 1997).

The induction factor preproteins resemble bacteriocin preproteins because they have leader peptides of the double glycine-type. These leader peptides are probably involved in excretion by the same ABC transporter and accessory proteins that are also involved in bacteriocin secretion (Nes, 1996; Kleerebezem et al., 1997). Both types of molecules resemble each other in that they are partly amphiphilic and adopt helical structures in membrane-mimicking environments (Eijsinck et al., 1996). In an early report on plantaricin production by *L. plantarum* C11, these similarities led to the erroneous description of an induction factor as the bacteriocin plantaricin A (Nissen-Meyer et al., 1993b). It was subsequently shown that synthetically produced plantaricin A did not have bacteriocin activity (Diep et al., 1995). Induction factors can be distinguished from bacteriocins in that they are generally shorter, ranging from 19 to 26 amino acids (Table 1.4). Some of the induction factors also share common features. For example, a C-terminal sequence of nine amino acids appears to be conserved. This sequence is flanked by two cysteines which may form a disulfide bridge (Nilsen et al., 1998) (Table 1.4). The induction factors associated with bacteriocin production include: EntF produced by *E. faecium* CTC492 (Nilsen et al., 1998); CbaX from *C. piscicola* LV17A (Worobo, 1996); CbnS from *C. piscicola* LV17B (Quadri et al., 1997); and the induction factors or putative induction factors encoded by *orf1* in *L. sake* strains Lb674 and LTH673 (Eijsinck et al., 1996; Brurberg et al., 1997) and by *orfX* in *L. sake* Lb706 (Axelsson and Holck, 1995). In the case of bacteriocin production by *C. piscicola* LV17B

it appears that in addition to EntS the bacteriocin CbnB2 can induce its own production (Saucier et al., 1995; Quadri et al., 1997).

Table 1.4 Amino acid sequences of induction factors and putative induction factors.

Class II bacteriocin system	Induction factor gene	Amino acid sequence	Reference
Carnobacteriocin A	*cbaX*	SINSQIGKATSSISKCVFSFFKKC	Worobo, 1996
Carnobacteriocin B2	*cbnS*	SKNSQIGKSTSSISKCVFSFFKKC	Quadri et al., 1997
Enterocins A, B	*entF*	AGTKPQGKPASNLVECVFSLFKKCN	Nilsen et al., 1998
Plantaricins JK, EF and N	*plnA*	KSSAYSLQMGATAIKQVKKLFKKWGW	Diep et al., 1995, 1996
Sakacin A	*orfX*	TNRNYGKPNKDIGTCIWSGFRHC	Axelsson and Holck, 1995
Sakacin P	*orf1*	MAGNSSNFIHKIKQIFTHR	Eijsinck et al., 1996; Brurberg et al., 1997
Consensus		GK S CVFSFFKKC	Nilsen et al., 1998

The transcription of genes involved in the production of class II bacteriocins was extensively analyzed in *L. plantarum* C11, *C. piscicola* LV17B and *L. sake* LTH673 and several promoters were mapped within the gene clusters involved (Fig. 1.5). All of the promoters appear to be regulated and sequence alignment reveals the presence of direct repeats of nine or ten nucleotides that are separated by 12 to 14 nucleotides, which are located two to nine base pairs upstream of the –35 site. These conserved, regulatory-like boxes (Diep et al., 1996, Nes et al., 1996) are probably the binding sites for the response regulators (Diep et al., 1996; Nes et al., 1996; Kleerebezem et al., 1997). Transcription regulators often bind as dimers to target DNA repeats, attaching their binding sites one subunit on each repeat (Diep et al., 1996). Regarding the likely role of the response regulators as transcription regulators, Diep et al. (1996) noticed a high occurrence of basic residues at the C terminus of these molecules which may interact with the negatively charged phosphate groups on the DNA backbone (Diep et al., 1996).

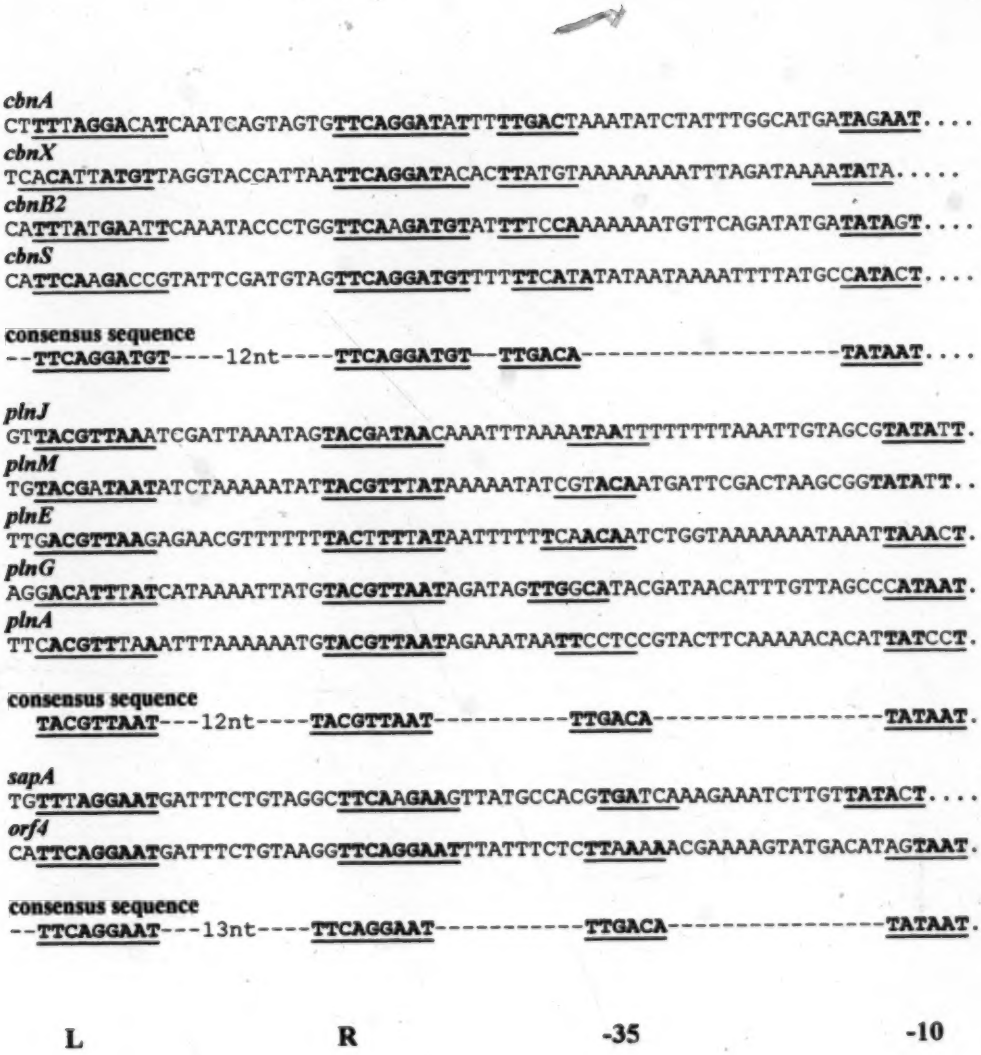

Figure 1.5 Alignment of promoter and regulatory-like box sequences from carnobacteriocins, plantaricins and sakacin P and their respective induction factor genes. The consensus sequence for each bacteriocin system is shown, with left (L) and right (R) direct repeats, as well as –35 and –10 promoter sequences. Data summarized from Nes et al., 1996; Saucier et al., 1997; and Quadri et al., 1997.

It is not exactly known how bacteriocin synthesis is induced. One model assumes that induction factors are constitutively produced in low amounts leading to a gradual accumulation during growth. When the concentration exceeds a threshold level for autoinduction of the induction factor, bacteriocin production will follow. This model relies on a cell-density dependent regulation (Nes et al., 1996; Kleerebezem et al., 1997). However, bacteriocin regulation by several LAB does not appear to fulfill this requirement, which may suggest the involvement of other (environmental) factors (Kleerebezem et al., 1997). An alternative model assumes that constitutive production of the induction factor is at a concentration just below the activation threshold for induction. Changes in environmental conditions (e.g., changes in nutrient conditions in the growth medium) may cause a short but temporary increase in induction factor production which leads to its autoinduction and subsequently to bacteriocin production (Nes et al., 1996; Kleerebezem et al., 1997). This model implies a growth condition-dependent phenotype (Kleerebezem et al., 1997). To date there is no experimental data that supports either of the models (Nes et al., 1996).

1.2.4. *Mode of action of class II bacteriocins.*

Class II bacteriocins act on the cytoplasmic membrane of sensitive cells thereby dissipating ion gradients and the proton motive force (Abee et al., 1995; Jack et al., 1995). They act on sensitive cells regardless of their degree of prior energization, suggesting that the loss of permeability of the cytoplasmic membrane occurs in a voltage-independent manner (Abee, 1995; Abee et al., 1995; Jack et al., 1995). For some bacteriocins it is believed that a membrane receptor in the sensitive cell is required for bacteriocin binding and subsequent insertion into the membrane (Abee, 1995). For example, the specificity of lactococcin A for lactococci is thought to result from interaction with a *Lactococcus*-specific membrane receptor protein (van Belkum et al., 1991). Evidence for such a receptor was based on the fact that treatment with proteinase K rendered membrane vesicles insensitive to the bacteriocin as a result of digestion of the receptor (Venema et al., 1994). Also, the receptor protein is thought to be involved in pore formation. Lactococcin A could not permeabilize liposomes composed only of

phospholipids obtained from sensitive lactococcal cells (van Belkum et al., 1991). Similarly, studies by Chikindas et al. (1993) suggested pediocin PA-1 forms hydrophilic pores in the cytoplasmic membranes of target cells in a protein receptor-mediated, voltage-independent manner (Chikindas et al., 1993). In contrast, thermophilin 13 has a broad activity spectrum, and dissipates the membrane potential and the pH gradient in liposomes from sensitive strains, which suggested that a membrane receptor is not required for activity (Marciset et al., 1997). Plantaricin C similarly acts on the cytoplasmic membrane as well as liposomes, indicating that this bacteriocin also does not require a specific receptor in the target membrane (González et al., 1996).

A general model of pore formation by class II bacteriocins involves bacteriocin binding by a proteinaceous receptor (when applicable), proton motive force-independent insertion of the bacteriocin into the membrane and formation of a pore by aggregation of bacteriocin monomers by a barrel-stave mechanism (Klaenhammer, 1993; Abee et al., 1995; Jack et al., 1995). In this process, aggregation of peptide monomers occurs in the membrane with the hydrophobic sides facing the membrane and the hydrophilic sides facing the pore (Klaenhammer, 1993).

1.2.5. *Applications of class II bacteriocins from lactic acid bacteria in food.*

During recent years, the production *in situ* of some class II bacteriocins by LAB has been studied in several foods and model food systems (Holzapfel et al., 1995; Schillinger et al., 1996), but it is commonly stated that except for nisin, applied studies on bacteriocins are lacking (Stiles, 1996). This may be understandable because no other bacteriocin has yet been approved for addition to foods (Nettles and Barefoot, 1993; Stiles, 1996). Yet it is clear that class II bacteriocins are present in the food supply. Two commercial compounds that have been licensed for addition to foods are Microgard and Alta 2341. However, these are ferments of food grade bacteria that impart antibacterial properties to the foods (Stiles, 1996), in contrast to nisin, which is used as a purified substance.

Applications of pure bacteriocin, bacteriocin-producing cultures or bacteriocin-containing ferment from a variety of LAB have been studied in meat, fish, dairy and

vegetable foods or model food systems (Holzapfel et al., 1995; Muriana, 1996; Schillinger et al., 1996). Part of the research in our laboratory is focused on the application of bacteriocinogenic LAB cultures for the preservation of packaged meat products, specifically ground beef. Aerobic storage of fresh meats at low temperatures leads to a relatively rapid and 'putrefactive'-type of spoilage in which the spoilage flora is dominated by the psychrotrophic, gram-negative bacteria belonging to the genera *Pseudomonas, Acinetobacter* and *Psychrobacter*. The shelf life of fresh meats can be greatly extended by vacuum or modified atmosphere packaging in combination with low storage temperatures. In this case, the spoilage microflora is dominated by gram-positive LAB belonging to the genera *Carnobacterium, Lactobacillus, Leuconostoc* and *Weissella* (Dainty and Mackey, 1992; Borch et al., 1996; Stiles and Holzapfel, 1997). Thus, although the LAB and their fermentation products can preserve foods, their adventitious growth in foods can also cause spoilage. In meats, the products of the adventitious fermentation are less noticeable, yet at some (unpredictable) time after the maximum LAB population is achieved, the meat spoils due to accumulated metabolic end-products (Stiles, 1996). The reason for this 'hidden' fermentation relies on the facts that the low carbohydrate content and the strong buffering capacity of meat do not produce as dramatic sensory changes as are the case for milk or vegetable fermentations (Stiles, 1996).

The spoilage characteristics of packaged fresh meats include 'sour' or 'acid' odors which arise from the acid end-products of LAB metabolism; these odors are less offensive than the putrid odors associated with spoiled, aerobically stored meats (Dainty and Mackey, 1992). The relatively inoffensive spoilage symptoms caused by LAB, the production of bacteriocins by LAB strains which are naturally associated with meats and the spoilage of meats after the maximum LAB population is reached make application of bacteriocin-producing starter cultures as biopreservative agents an attractive opportunity for prolonging storage life. Molecular biological techniques involved with transfer of genetic material between LAB have developed rapidly in recent years. Shuttle vectors that replicate both in LAB and *E. coli* have become available and techniques for introduction of foreign DNA into LAB, for example by electrotransformation, have become well established techniques. These genetic tools also allow the genetic

modification of starter LAB that may be used in biopreservation, and genes associated with bacteriocin production from organisms that would not normally grow in a meat environment may be transferred to such starter LAB.

In a study in our laboratory chill stored, vacuum-packaged beef was inoculated with a sulfide-producing *L. sake* strain that develops a distinct sulphurous odor in the meat package within three weeks of storage at 2°C. Co-inoculation of the meat with a bacteriocinogenic strain of *L. gelidum* delayed the *L. sake*-mediated spoilage of meat for up to 8 weeks of storage (Leisner et al., 1996), clearly demonstrating that application of bacteriocin-producing LAB is a viable strategy for prolonging the shelf life of vacuum-packaged fresh meat.

Use of bacteriocin-producing starter cultures may also be of advantage in controlling foodborne pathogens. Many class II bacteriocins have anti-*Listeria* activity and suppression of this pathogen by bacteriocinogenic starter culture is an attractive argument for development of biopreservation technology. Bacteriocin-producing LAB have been used successfully in application-type studies to inhibit the pathogen *Listeria monocytogenes* (Muriana, 1996; Stiles, 1996; McMullen and Stiles, 1996). Enterococci are microorganisms of intestinal origin that can contaminate meats. These microorganisms have recently received much attention because of their association with nosocomial disease as well as their resistance to almost every antibiotic currently in use. Recently, vancomycin resistant enterococci have been isolated from the food supply, which raises questions about the role of these bacteria in foods, and whether there is a potential for dissemination of potentially pathogenic enterococci in foods. On the other hand, the enterococci may produce bacteriocins which may be useful in biopreservation. As this thesis is mainly concerned with bacteriocin production by a strain of *Enterococcus faecium* isolated from food, the role of enterococci in foods and the implications of their presence in foods with respect to food safety will be reviewed below.

1.3. The enterococci.

1.3.1. *Phylogeny and taxonomy of enterococci.*

The enterococci as a group were first described by Thiercelin (1899), and the genus *Enterococcus* was proposed by Thiercelin and Jouhaud (1903) for gram-positive diplococci of intestinal origin. Andrewes and Horder (1906) classified potentially pathogenic bacteria from a patient with endocarditis as *Streptococcus faecalis*. Because of their close resemblance with strains isolated from the human intestine the species epithet 'faecalis' was suggested. Lancefield (1933) developed a serological typing system for streptococci in which those of 'fecal origin' possessed the group D antigen. This correlated with the grouping of Sherman (1937) who proposed a new classification scheme for the genus *Streptococcus* that separated it into four divisions designated: pyogenic, viridans, lactic and enterococcus. The enterococcus group included *Streptococcus faecalis, Streptococcus faecium, Streptococcus bovis* and *Streptococcus equinus* as the enterococcal or Lancefield group D strains. *'Streptococcus durans'* assumed various levels of acceptance, either as a separate species or as a subspecies of *S. faecium* within this group.

The classical taxonomy of the enterococci is vague because there are no phenotypic characteristics that unequivocally distinguish them from other gram-positive, catalase-negative, coccus-shaped bacteria (Devriese et al., 1993). The majority of *Enterococcus* species, however, can be distinguished from such cocci by their ability to grow at 10 and 45°C, in 6.5% sodium chloride, at pH 9.6 and to survive heating at 60°C for 30 min (Hardie and Whiley, 1997; Morrison et al., 1997). The genus *Enterococcus* was described by Schleifer and Kilpper-Bälz (1984), who used DNA:DNA and DNA:rRNA hybridization to show that *S. faecalis* and *S. faecium* were sufficiently distinct from other streptococci to warrant their transfer to a separate genus. Based on 16S rRNA cataloguing (Ludwig et al., 1985; Williams et al., 1991), DNA:DNA and DNA:rRNA hybridization (Garvie and Farrow, 1981; Kilpper-Bälz and Schleifer, 1981, 1984; Kilpper-Bälz et al., 1982; Schleifer and Kilpper-Bälz, 1984; Schleifer et al., 1985) and serological studies with superoxide dismutase antisera (Schleifer et al., 1985), the

streptococci *sensu lato* were subdivided into three genera: *Streptococcus sensu stricto, Enterococcus* and *Lactococcus* (Devriese et al., 1993).

Since 1984, chemotaxonomic and phylogenetic studies have resulted in assignment of 19 species to the genus *Enterococcus* (Devriese et al., 1993; Devriese and Pot, 1995; Hardie and Whiley, 1997; Stiles and Holzapfel, 1997). Based on 16S rRNA sequences, the presence of four 'species groups' within the genus was revealed, with the 'faecium' group comprising *E. faecium, E. durans, E. hirae* and *E. mundtii*; the 'avium' group comprising *E. avium, E. raffinosus, E. malodoratus* and *E. pseudoavium;* the 'gallinarum' group comprising *E. casseliflavus* and *E. gallinarum;* and the possibly related *E. columbae* and *E. cecorum* representing a fourth group (Williams et al., 1991; Devriese and Pot, 1995). All of the other enterococci described to date, i.e., *E. faecalis, E. dispar, E. flavescens, E. saccharolyticus, E. sulfureus* and *E. seriolicida,* form individual lines of descent (Devriese et al., 1993; Devriese and Pot, 1995).

1.3.2. *Environmental sources and food contamination.*

Enterococci constitute a large proportion of the autochtonous bacteria associated with the mammalian gastrointestinal tract. *E. faecalis* is often the predominating *Enterococcus* spp. in the human bowel, although in some individuals and in some countries, *E. faecium* outnumbers *E. faecalis* (Ruoff, 1990; Devriese and Pot, 1995). Numbers of *E. faecalis* in human feces range from 10^5 to 10^7/g compared with 10^4 to 10^5/g for *E. faecium* (Noble, 1978). *E. faecalis,* but not *E. faecium*, has been isolated from the feces of neonates (Noble, 1978).

Although *E. faecalis, E. faecium* and *E. durans* are frequently isolated from human feces, they are much less prevalent in livestock such as pigs, cattle and sheep (Leclerc et al., 1996). In a study by Devriese et al. (1992), *E. faecalis* was isolated from feces of pre-ruminant calves and ruminating young cattle and dairy cows, and *E. faecium* from pre-ruminant calves, but not from ruminating young cattle or dairy cows. *S. bovis* was the predominant group D organism isolated from feces of dairy cows. *E. faecalis, E. faecium, E. hirae* and *E. cecorum* were the enterococci most frequently isolated from pig intestines, while *E. faecium* predominated in fecal samples (Devriese et al., 1994; Leclerc

et al., 1996). The intestinal microflora of young poultry contained principally *E. faecalis* and *E. faecium*, but *E. cecorum* predominated in the intestine of chickens over 12 weeks old (Devriese et al., 1991). Enterococci are not only associated with warm-blooded animals, but they also occur in soil, surface waters and on plants and vegetables (Mundt, 1961, 1963; Niemi et al., 1993; Jay, 1996; Leclerc et al., 1996). *E. faecium* is also among the predominant microorganisms in raw milk (Devriese and Pot, 1995), which has important implications for the dairy industry.

1.3.3. *Enterococci in the food supply.*

1.3.3.1. *Association of enterococci with meats.* The presence of enterococci in the gastrointestinal tract of animals leads to a high potential for contamination of meat at the time of slaughter. In a study of enterococci from raw meat products, *E. faecalis* was the predominant isolate from beef and pork cuts (Stiles et al., 1978). *E. faecium* was also frequently isolated from a processed meat sausage (Stiles et al., 1978). Pig carcasses from three different slaughtering plants contained mean log counts of 10^4 to 10^8 enterococci per 100 cm^2 of carcass surface throughout processing, and *E. faecium* and *E. faecalis* were the most predominant *Enterococcus* spp. isolated (Knudtson and Hartman, 1993a). *E. faecalis* predominated the gram-positive coccal species isolated from chicken samples collected at poultry abattoirs (Turtura and Lorenzelli, 1994). Fermented meat products such as Salami and Landjäger types contain enterococci at numbers ranging from 100 to 2.6×10^5/g (Teuber et al., 1996).

Enterococci are among the most thermotolerant of the non-sporulating bacteria (Sanz Perez et al., 1982). Because of this, they can become a spoilage problem in cooked, processed meats. Processed meats are typically salted or cured, and either raw or cooked (Tompkin, 1986). Cooking of processed meats raises the core temperature of products to at least 60°C and frequently above 70°C (Carr and Marchello, 1986). After surviving heat processing, both *E. faecalis* and *E. faecium* have been implicated in spoilage of cured meat products such as pasteurized canned hams and chub-packed luncheon meats (Bell and Gill, 1982; Houben, 1982; Bell and DeLacey, 1984; Magnus et al., 1986). This is

especially true where recontamination with competing bacteria is prevented when products are heated after packaging in cans or in impermeable plastic films (Bell and DeLacey, 1984). The heat resistance of enterococci in these products is influenced by components such as salt, nitrite and meat tissue (Houben, 1982; Bell and DeLacey, 1984; Magnus et al., 1986, 1988). To prevent spoilage of the processed meats by enterococci, it was suggested that initial contamination by these microorganisms should be kept to a minimum, and that adequate heat processing should be based on D-values of the most heat resistant enterococci isolated from raw materials (Magnus et al., 1986; André Gordon and Ahmad, 1991).

In spoilage of vacuum-packaged processed meats, members of the genera *Lactobacillus* and *Leuconostoc* usually predominate, but they are often accompanied by varying proportions of enterococci and pediococci (Reuter, 1981; Holzapfel and Gerber, 1986; von Holy et al., 1991). These products are usually surface contaminated with LAB after heat treatment and before packaging (Dykes et al., 1991). This may explain why other LAB and not enterococci usually predominate in the spoilage of these products. Higher levels of enterococci in processed meats may result from the practice known as 'reworking', whereby meat from faulty products (e.g., in which the packaging material was damaged during heat treatment) is added to the raw materials for further processing. If the faulty product contained heat resistant enterococci, these may multiply during reworking and survive a second heat processing step in greater numbers. Because of this high heat resistance and survival under adverse environmental conditions, the enterococci have frequently been suggested as indicators of sanitary quality of food (Jay, 1996).

The role of the meat chain in the transfer of vancomycin-resistant enterococci (VRE) has not been fully elaborated. In a study of 555 samples of 115 batches of minced beef and pork, Klein et al. (1998) found an incidence of only 0.5% VRE-positive samples, at a concentration of 1.0 log CFU/g, when a direct plating method was used. They also found that the antibiotic resistance patterns of VRE isolates differ from those of clinical isolates. Nevertheless, a great potential exists for contamination of meat products with enterococci from intestinal or environmental sources. Effective control of meat contamination by enterococci may become more important in the future with increasing recognition of these bacteria as opportunistic human pathogens.

1.3.3.2. *The role of enterococci in cheese manufacture.* Enterococci occur and grow in a variety of cheeses, especially artisanal cheeses produced in southern Europe (Portugal, Spain, Italy and Greece) from raw or pasteurized goat, ewe's, water-buffalo or cow milk. High levels of contaminating enterococci usually result from poor hygienic practices during cheese manufacture (Thompson and Marth, 1986; Litopoulou-Tzanetaki, 1990; López-Díaz et al., 1995) and lead to deterioration of sensory properties in some cheeses (Thompson and Marth, 1986; López-Díaz et al., 1995), but they play a major role in ripening and aroma development in other cheeses (Ordoñez et al., 1978; Trovatelli and Schiesser, 1987; Coppola et al., 1988; Litopoulou-Tzanetaki, 1990; Torri Tarelli et al., 1994; Macedo et al., 1995; Centeno et al., 1996). Levels of enterococci in different cheese curds range from 10^4 to 10^6/g, and in the fully ripened cheeses from 10^5 to 10^7/g (Table 1.5). *E. faecium* and *E. faecalis* were the dominant enterococcal isolates in these cheeses. Varying levels in different cheeses result from the extent of milk contamination and survival in the dairy environment (dependent on seasonal temperature), as well as survival and growth under the particular conditions of cheese manufacture and ripening (Litopoulou-Tzanetaki, 1990; Litopoulou-Tzanetaki and Tzanetakis, 1992; Macedo et al., 1995). In some cheeses, e.g., Cebreiro, Kefalotyri, Manchego, Picante da Beira Baixa, and Teleme (Table 1.5), enterococci are also the predominant microorganisms in the fully ripened product. In other traditional cheeses, LAB such as *Lactobacillus plantarum, Weissella* (previously *Leuconostoc*) *paramesenteroides, Leuconostoc lactis* or *Leuconostoc paracasei* predominate in the ripened product. However, the enterococci represent an important part of the bacterial flora of ripened cheeses (Table 1.5).

The dominance or persistence of enterococci in some cheeses during ripening can be attributed to their wide range of growth temperatures, their high tolerance of heat, salt and acid (Ordoñez et al., 1978; Litopoulou-Tzanetaki, 1990; Wessels et al., 1990; Litopoulou-Tzanetaki and Tzanetakis, 1992; Freitas et al., 1995) and their production of proteolytic enzymes involved in casein degradation (Trovatelli and Schiesser, 1987; Wessels et al., 1990). Salt concentration increases during cheese ripening. This is an important selection factor for growth of salt tolerant enterococci, *L. plantarum* and *W. paramesenteroides* during the late stages of cheese ripening (Ordoñez et al., 1978;

Table 1.5 Numbers and predominance of *Enterococcus* spp. in cheeses from Mediterranean countries

Cheese	Country Of origin	Milk Source	Enterococci in curd (log CFU/g)	Enterococci at end of ripening (CFU/g)	Predominant bacteria in end product (% of isolates) [b]	Reference
White-brined cheese	Greece	raw goat milk or mixed goat and ewes' milk	4.0	6.7	*L. plantarum* (47%) *E. faecium* (12%) *L. paracasei* subsp. *paracasei* (10%) *E. faecalis* (9%)	Litopoulou-Tzanetaki and Tzanetakis (1992)
Kefalotyri cheese	Greece	ewes' milk, cow milk or mixed ewes' and goat milk	4.9	5.8	*E. faecium* (35.6%) *L. plantarum* (18.4%) *L. casei* subsp. *casei* (15.8%) *E. durans* (9.2%) Pediococci (9.2 %)	Litopoulou-Tzanetaki (1990)
Teleme cheese	Greece	Pasteurized ewes' milk	n.r.[a]	n.r.	Lactobacilli Leuconostocs Enterococci	Tzanetakis and Litopoulou-Tzanetaki (1992)
La Serena ewe's milk cheese	Spain	raw ewes' milk	6.2	7.2	Lactobacilli Leuconostocs Enterococci	Del Pozo et al. (1988)
Manchego cheese	Spain	raw ewes' milk	n.r.	n.r.	Enterococci	Ordoñez et al. (1978)
Cebreiro	Spain	raw cow milk	n.r.	6.5	*E. faecalis* (30.1%) *E. faecalis* (var *liquifaciens*) (11.9%) *Lact. lactis* (19.0%) *W. (Leuc.) paramesenteroides* (7.9%) *Leuc mesenteroides* subsp. *mesenteroides* (6.3%) *E. faecium* (4.8%)	Centeno et al. (1996)
Serra cheese	Portugal	raw ewes' milk	n.r.	n.r.	*Leuc. lactis, Lact. lactis, Leuc. mesenteroides* subsp. *mesenteroides/dextranicum* *E. faecium*	Macedo et al. (1995)
Picante da Beira Baixa cheese	Portugal	mixture of raw goat and ewes' milk	n.r.	n.r.	*E. faecium, E. faecalis, E. durans, L. plantarum, L. paracasei*	Freitas et al. (1995)

[a] n.r. = not reported; [b] : *L. = Lactobacillus; E. = Enterococcus; Lact. = Lactococcus; Leuc. = Leuconostoc; W. = Weissella*

Litopoulou-Tzanetaki, 1990; Freitas et al., 1995). Enterococci show higher proteolytic activity than other LAB and this is considered important for cheese ripening (Ordoñez et al., 1978; Trovatelli and Schiesser, 1987; Centeno et al., 1996). The beneficial effect of enterococci in cheese making has also been attributed to hydrolysis of milk fat by esterases (Tsakalidou et al., 1993). In addition, enterococci produce typical flavour components such as acetaldehyde, acetoin and diacetyl (Trovatelli and Schiesser, 1987, Centeno et al., 1996). This beneficial role of enterococci in development of cheese aroma has led to inclusion of enterococcal strains in certain starter cultures. For example, enterococci were suggested for use as a starter culture in production of Cebreiro cheese (Centeno et al., 1996). Similarly, *E. durans* was shown to be important for aroma development in Feta cheese when used in a starter together with other LAB (Litopoulou-Tzanetaki et al., 1993). For Mozzarella cheese made from raw water-buffalo milk, a strain of *E. faecalis* was selected together with other LAB for use in a starter culture preparation (Coppola et al., 1988; Parente et al., 1989). Clearance was sought by the British 'Advisory Committee on Novel Foods and Processes' (ACNFP) for the use of *E. faecium* strain K77D as a starter culture in fermented dairy products (ACNFP, 1996). Clearly, the enterococci play an important role in the manufacture of cheeses typical of some regions, and their use has a major impact on this part of the dairy industry.

1.3.4. *Enterococcal bacteriocins: the enterocins.*

Strains of enterococci, including *E. faecium* and *E. faecalis*, are known to produce bacteriocins (Table 1.6). These are generally called enterocins and they mainly belong to the class II bacteriocins. Examples of the best characterized enterocins are enterocin A, a class IIa, pediocin-like bacteriocin (Aymerich et al., 1996), and enterocin B (Casaus et al., 1997; this thesis), a bacteriocin that is not pediocin-like, but it is considered to belong to the class IIa bacteriocins together with enterocin A because of its chemical characteristics, heat stability and anti-*Listeria* activity (Table 1.6). Enterocin P belongs to class IIc because secretion occurs by the *sec*-pathway (Cintas et al., 1997). Enterocins L50A and L50B are novel bacteriocins (Cintas et al., 1998). Each has antimicrobial activity on its own, but together they show synergistic activity (Table 1.6).

Table 1.6 Well characterized bacteriocins produced by *E. faecium* and *E. faecalis*.

Bacteriocin	Producer organism	Location of genes for production	Number of amino acids: Prepeptide	Number of amino acids: Mature peptide	Molecular mass (Da)	Posttranslational modification	Reference
Enterocin A	*E. faecium*	chromosome	65	47	4829	Cleavage of leader peptide on export, possible disulfide bridge formation	Aymerich et al. (1996)
Enterocin B	*E. faecium*	chromosome	71	53	5463	Cleavage of leader peptide on export, possible disulfide bridge formation	This study, Casaus et al. (1997)
Enterocin P	*E. faecium*	chromosome	71	43	4493	Cleavage of leader peptide on export, possible disulfide bridge formation	Cintas et al. (1997)
Enterocin L50A	*E. faecium*	plasmid pCIZ1	n.a.	44	5190	No modification	Cintas et al. (1998)
Enterocin L50B	*E. faecium*	plasmid pCIZ1	n.a.	43	5178	No modification	Cintas et al. (1998)
Bacteriocin 31	*E. faecalis*	pheromone responsive plasmid pYI17	67	43	n.r.	Cleavage of signal peptide on export, possible disulfide bridge formation	Tomita et al. (1996)
AS-48	*E. faecalis*	pheromone responsive plasmid pMB2	105	70	n.r.	Cleavage of leader peptide and head-tail peptide bond formation	Martínez-Bueno et al. (1994)
Cytolysin:	*E. faecalis*	pheromone responsive plasmid pAD1				Modification to form lanthionine and β-methyllanthionine- containing precursors, removal of a leader sequence on export and proteolytic activation	Booth et al. (1996)
$CylL_L$'' peptide			68	38	3437		
$CylL_S$'' peptide			63	21	2031		

n.r. not reported, n.a. not applicable

They are not posttranslationally modified and do not require the presence of a leader or signal peptide for excretion (Cintas et al., 1998). They share homology with the staphylococcal hemolysins, yet they do not show hemolytic activity.

Bacteriocins of some strains of *E. faecalis* are interesting because they are encoded on pheromone-responsive, conjugative plasmids, for example AS-48, cytolysin and bacteriocin 31 (Huycke et al., 1992; Martínez-Bueno et al., 1994; Booth et al., 1996; Tomita et al., 1996) (Table 1.6). The antimicrobial peptide AS-48 produced by *E. faecalis* AS-48 is similar to bacteriocins because it functions by permeabilizing the cytoplasmic membrane of susceptible bacteria (Martínez-Bueno et al., 1994). Unlike most other bacteriocins, this peptide is a cyclic molecule that results from posttranslational modification of the primary product during secretion (Martínez-Bueno et al., 1994). In addition, strains of *E. faecalis* produce hemolysin/bacteriocins, which are two component bacteriocins with both hemolytic and bacteriocin activity. These bacteriocins are called cytolysins and contain lanthionine and β-methyllanthionine residues (Booth et al., 1996), suggesting that they are class I bacteriocins. Bacteriocin 31 produced by *E. faecalis* YI717 is similar to enterocin P, because it is secreted by the *sec*-pathway (Tomita et al., 1996). The enterococci produce a wider variety of antimicrobial peptides than has been described for strains of most other LAB genera. Although bacteriocin production has been reported for many other strains of *E. faecalis*, most of these have either not been purified or they are insufficiently characterized for classification as cytolysins or class I, II or III bacteriocins (Arihara et al., 1991; Villani et al., 1993; Maisnier-Patin et al., 1996; Simonetta et al., 1997). Using a semi-nested PCR assay for the AS-48 structural gene, Joosten et al. (1997) showed that production of bacteriocins very similar or identical to AS-48 is common in *E. faecalis* and *E. faecium*.

The enterocins are generally active against other enterococci as well as strains of *Listeria monocytogenes* (Giraffa, 1995). The anti-*Listeria* activity may be explained by the fact that enterococci and listeriae are phylogenetically closely related (Devriese and Pot, 1995). Some enterocins are active against other LAB as well as *Clostridium* spp., including *C. botulinum*, *C. perfringens* and *C. tyrobutyricum* (Torri Tarelli et al., 1994; Franz et al., 1996). Bacteriocinogenic enterococci have been isolated from a variety of

sources, including fermented meat (Aymerich et al., 1996; Casaus et al., 1997), dairy products (Olasupo et al., 1994; Torri Tarelli et al., 1994; Vlaemynck et al., 1994; Farías et al., 1996), and vegetables (McKay, 1990; Villani et al., 1993; Franz et al., 1996). Bacteriocinogenic enterococci may be used as anti-*Listeria* agents in the dairy industry, particularly in certain types of soft cheese (e.g., Camembert or Taleggio) where pH in the rind increases to a level that allows growth of *L. monocytogenes.* Enterococci often predominate during ripening of cheeses and could produce bacteriocins at sufficient levels to inhibit *Listeria* (Giraffa et al., 1997). Bacteriocinogenic *E. faecium* was tested on a laboratory scale for use in combination with a commercial starter culture for Taleggio cheese making (Giraffa et al., 1995). Bacteriocin was produced during drainage of the whey and activity could be detected in the cheese until the end of the ripening period, while growth and acidifying activity of the thermophilic commercial starter culture was not inhibited (Giraffa et al., 1995). As a further example, an inhibitory starter culture consisting of bacteriocinogenic *E. faecium, E. faecalis,* nisin-producing *L. lactis* and *Lactobacillus paracasei* added to milk prior to Camembert cheese making or sprayed onto the surface of the cheese, totally inhibited *Listeria* spp. when surfaces were contaminated with *Listeria* not later than 1.5 days after brining (Sulzer et al., 1992).

Activity of enterococci against *C. tyrobutyricum* has been described. This may be used to preserve packaged cheeses where growth of *C. tyrobutyricum* causes blowing of the packages. Inhibition of these bacteria by enterocins would eliminate this defect. Use of bacteriocinogenic enterococci as starter cultures for cheese manufacture to increase cheese safety or to prolong storage life of the product has not yet been practiced on an industrial scale. It is conceivable that the food industry may pursue application of bacteriocin-technology to safeguard cheese from growth of pathogens, to increase storage life of cheese and to reduce economic loss.

1.3.5. *Enterococci as probiotics.*

Probiotics may be defined as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 1989). Products for human consumption containing probiotic organisms may be grouped into

three categories: infant foods, cultured milks and pharmaceutical preparations (O'Sullivan et al., 1992). Claims for beneficial effects include: maintenance/restoration of the normal intestinal microflora and thereby prevention or reduction of gastrointestinal tract disorders, alleviation of lactose intolerance, reduction in serum cholesterol levels, anticarcinogenic activity, stimulation of the immune system and improved nutritional value of foods (Fuller, 1989; O'Sullivan et al., 1992; Agerbaeck et al., 1995; Lee and Salminen, 1995; Salminen et al., 1996).

Although both *E. faecium* and *E. faecalis* find application in probiotic preparations for humans, *E. faecalis* is more widely used as an animal feed supplement. *E. faecium* strain SF68 has been studied in great detail for use as a human probiotic, especially in the treatment of diarrhea. The strain was originally isolated in Sweden and was patented in Switzerland and other countries (Lewenstein et al., 1979). Its effectiveness for treatment of intestinal disorders can probably be attributed to the fact that it is a commensal of the intestine and that it has a short lag phase and generation time (*ca.* 20 min under optimal conditions). It is moderately resistant to antibiotics and it has an inhibitory effect *in vitro* against growth of *E. coli, Salmonella* spp., *Shigella* spp. and *Enterobacter* spp. In addition, this strain is resistant to low pH, insensitive to bile salts, and individuals show a high tolerance to it with no side effects (Lewenstein et al., 1979; Bellomo et al., 1980; Canganella et al., 1996). Use of *E. faecium* SF68 for treatment of diarrhea is considered an alternative to antibiotic treatment (Lewenstein et al., 1979; Bellomo et al., 1980). Clinical studies have shown the effectiveness of *E. faecium* in the treatment of enteritis in both children (Bellomo et al., 1980; D'Apuzzo and Salzberg, 1982) and adults (Lewenstein et al., 1979; Bruno and Frigerio, 1981). These controlled, 'double blind' studies on the treatment of enteritis with SF68 showed a statistically significant decrease in the duration of diarrhea and time for normalization of stools (Bellomo et al., 1980; Bruno and Frigerio, 1981; D'Apuzzo and Salzberg, 1982). However, in one study with *E. faecium* SF68 no anti-diarrheal effect was demonstrated in adults suffering from acute diarrhea due to *Vibrio cholerae* or enterotoxinogenic *E. coli* infections (Mitra and Rabbani, 1990). In another controlled study it was shown that *E. faecium* SF68 significantly decreased the incidence of diarrhea and prevented mucositis (vitamin deficiency) in chronic pulmonary tuberculosis patients receiving long-term

antibiotic treatment, so that supportive treatment with an *E. faecium* probiotic preparation was recommended for these patients (Borgia et al., 1982).

Some enterococcal probiotic preparations are sold in 'health food' stores as nutrition supplements, with vague claims to 'improve the gastrointestinal balance'. Sufficient scientific information on certain probiotic preparations from health food stores is not always available. Clearly, at a time when consumer awareness of nutrition and health is increasing, it is important that strains used in probiotic preparations to promote human health should be safe for all potential consumers.

1.3.6. *Enterococci in disease.*

Enterococci are considered as emerging pathogens of humans, and their role and importance have been reviewed by Murray (1990), Lewis and Zervos (1990) and Morrison et al. (1997). They have become of major importance in community-acquired and in hospital-acquired (nosocomial) infections and superinfections such as endocarditis, bacteremia, urinary tract, neonatal, central nervous system (CNS), intraabdominal and pelvic infections. In the U.S.A. their importance has increased from the third leading cause of nosocomial disease, accounting for 10% of such infections in 1984 (Chenoweth and Schaberg, 1990), to the second between 1985 and 1989 (Schaberg et al., 1991). *E. faecalis* clearly predominates among enterococci isolated from human infections, while strains of *E. faecium* are associated with the majority of the remaining infections (Jett et al., 1994).

Bacteremia is the most common form of enterococcal infection (Lewis and Zervos, 1990). Conditions associated with enterococcal bacteremia include underlying disease, presence of urethral or intravascular catheters, surgery, major burns, multiple trauma or prior antibiotic therapy (Lewis and Zervos, 1990). Enterococci cause an estimated 5 to 15% of cases of bacterial endocarditis with *E. faecalis* more commonly involved than *E. faecium* (Murray, 1990). Endocarditis often occurs in patients that had preceding genitourinary instrumentation or urinary tract infections (UTI), abortion, or urinary tract instrumentation (Chenoweth and Schaberg, 1990; Murray, 1990). Urinary tract infections are commonly caused by enterococci, especially in hospitalized patients.

In a 1984 report, a national nosocomial surveillance study in the U.S.A. listed enterococci as the third most common cause of nosocomial UTI's, occurring especially in persons who had been medically instrumented, received antibiotics, had structural abnormalities, or had recurrent enterococcal infections (Chenoweth and Schaberg, 1990; Murray, 1990). Enterococci account for as many as 13% of cases of neonatal sepsis and meningitis (Lewis and Zervos, 1990). Enterococci causing neonatal infection are thought to originate from the vagina, because they are detected in the vaginal microflora in 25% of healthy women (Lewis and Zervos, 1990). *E faecium* and *E. faecalis* have been implicated in outbreaks of neonatal infections, especially neonatal meningitis and CNS infections, although infections of older children and adults have also been reported. Most cases appear to be related to an underlying disease (Murray, 1990). Enterococci may cause or contribute to abdominal and pelvic abscess formation and sepsis (Murray, 1990). They were reported as a cause of spontaneous peritonitis in cirrhotics and nephrotics, and may be associated with peritonitis in patients on peritoneal dialysis (Murray, 1990).

The use of antibiotics to which enterococci are resistant is an important factor in enterococcal superinfection (Moellering, 1982; Zervos et al., 1988; Murray, 1990). Antibiotic resistance of enterococci and gene transfer mechanisms, which often involve antibiotic resistance, are discussed below; however, antibiotic resistance alone cannot explain the virulence of these bacteria.

1.3.7. *Antibiotic resistance.*

The enterococci have become a focus of attention in hospitals because of their increasing resistance to antibiotics. Antibiotic resistance and nosocomial infection are mutually reinforcing phenomena because resistance allows enterococci to survive in the hospital environment where antibiotics are used, and the hospital provides the opportunity for dissemination of resistant organisms (Murray, 1990). Examples of intrinsic resistance include the cephalosporins, β-lactams, sulphonamides, and low levels of clindamycin and aminoglycosides (Moellering, 1990; Murray, 1990; Leclercq, 1997; Morrison et al., 1997). Acquired resistance based on acquisition of plasmids and transposons, has relevance for chloramphenicol, erythromycin, high levels of

clindamycin and aminoglycosides, tetracycline, β-lactams (by β-lactamase or penicillinase), fluoroquinolones and glycopeptides (Murray, 1990; Moellering, 1991; Landman and Quale, 1997; Leclercq, 1997; Morrison et al., 1997).

Intrinsic resistance to many antibiotics suggests that treatment of infection could be difficult. However, combinations of cell-wall-active antibiotics with aminoglycosides (e.g., streptomycin, kanamycin and gentamicin) act synergistically and have been used successfully in treatment of enterococcal infection (Moellering, 1990, 1991; Murray, 1990). In the early 1970's, a high level of streptomycin resistance was reported, and strains were also found to be resistant to penicillin-streptomycin combinations. High level gentamicin resistance followed, with enterococci exhibiting resistance to combinations of penicillin and gentamicin (Moellering, 1990). In 1983 a strain of *E. faecalis* producing a β-lactamase identical to that produced by *S. aureus* was reported (Murray and Mederski-Samoraj, 1983), and it is believed that such *Enterococcus* strains received the gene for this from *S. aureus* (Murray et al., 1986). The hitherto successful penicillin-aminoglycoside treatment was no longer a viable option, resulting in a major therapeutic problem (Moellering, 1991).

Another major concern is the emergence of vancomycin-resistant enterococci (VRE). Different vancomycin resistance phenotypes are recognized in both *E. faecium* and *E. faecalis*. Acquired resistance phenotypes, which are transferable by conjugation, are the VanA and the VanB phenotypes (Arthur and Courvalin, 1993). The VanA-type confers high level and inducible resistance to both vancomycin and teicoplanin, while the VanB-type displays variable levels of inducible resistance only to vancomycin. Vancomycin was previously used for treatment of enterococcal infections with strains exhibiting high level β-lactam resistance, or when β-lactams were poorly tolerated by the patient (Leclercq et al., 1988; Shlaes et al., 1989). Unfortunately, many VRE are also highly resistant to all standard anti-enterococcal drugs, including penicillin-aminoglycoside combinations (Landman and Quale, 1997), and only a few alternatives remain for successful treatment (Fraise, 1996). Therefore, VRE presently constitute a serious risk group among bacterial nosocomial pathogens and their presence in hospitals is met with great concern.

Regarding the food chain, it is not clear whether and with what frequency VRE strains are transferred. Klein et al. (1998) could not demonstrate a direct connection between the occurrence of VRE in minced meat and nosocomial infections. On the other hand, by molecular characterization of resistance determinants for enterococci isolated from processed meat products and cheeses, Teuber et al. (1996) showed them either to be similar or identical to those from clinical samples.

1.3.8. *Gene transfer mechanisms in enterococci.*

Several gene transfer mechanisms in enterococci have been described. They involve both conjugative and nonconjugative plasmids as well as conjugative transposons, and these may carry antibiotic resistance genes (Clewell, 1990). Conjugative plasmids may have a broad bacterial host range and transfer at a low frequency in broth (e.g., pAMβ1 which has been transferred to lactococci, lactobacilli, *S. aureus* and *Bacillus* spp.), or narrow host range, such as the plasmids of *E. faecalis*, which transfer at a high frequency in broth and respond to sex pheromones (Clewell, 1990).

The sex pheromone response is an interesting system for highly efficient exchange of genetic material in *E. faecalis* (Dunny, 1990; Clewell, 1993; Wirth, 1994; Dunny et al., 1995). A plasmidless recipient strain produces chromosomally encoded pheromones which consist of hydrophobic peptides containing 7 to 8 amino acids. The pheromones induce genes on the plasmid of the donor strain to produce aggregation substance (AS), which facilitates binding to recipient cells by a complementary receptor on the recipient cell, called enterococcal binding substance (Dunny et al., 1995). A mating channel is formed and plasmid DNA is transferred from donor to recipient cell. The new recipient cell is prevented from responding to its own pheromone by a surface exclusion protein which is plasmid encoded (Dunny, 1990; Clewell, 1993; Wirth, 1994; Dunny et al., 1995). A strain of *E. faecalis* that harbors a specific sex pheromone plasmid will not secrete the corresponding pheromone; however, it may secrete pheromones specific for other sex pheromone plasmids which it does not possess but may subsequently acquire. It is not uncommon for strains of *E. faecalis* to harbor two or three sex pheromone plasmids (Wirth, 1994). Sex pheromone plasmids may carry one or more

antibiotic resistance genes, e.g., tetracycline, penicillin, gentamicin, streptomycin, kanamycin and tobramycin (Clewell, 1990; Wirth, 1994), or they may encode hemolysin/bacteriocin production.

Antibiotic resistance may contribute to an understanding of the establishment of enterococci as nosocomial pathogens. However, pathogenicity of enterococci can not be explained on the basis of antibiotic resistance alone, and virulence factors should also be taken into consideration in enterococcal diseases.

1.3.9. *Enterococcal virulence factors.*

For many years enterococci were considered to be harmless commensals with low pathogenic potential (Moellering, 1990; Jordens et al., 1994; Leclercq, 1997). In general terms this is true because they lack potent virulence factors compared with other gram-positive pathogens, such as pathogenic streptococci, *S. aureus* and *L. monocytogenes* (Moellering 1990). However, this view is changing because of the increasing role of enterococci in nosocomial infections, especially under selective pressure of antibiotics (Chenoweth and Schaberg, 1990; Leclercq, 1997). For enterococci to cause infection, they must colonize host tissue, resist host defense mechanisms (specific and nonspecific), and produce pathological changes (Jett et al., 1994; Johnson, 1994).

1.3.9.1. *Colonization.* It is assumed that enterococci are able to colonize the gastrointestinal tract because they are normal inhabitants of the human intestine. In addition, it has long been considered that enterococcal infections originate from the gastrointestinal and genitourinary tracts (Chenoweth and Schaberg, 1990; Murray, 1990; Johnson, 1994). Evidence exists for patient-to-patient spread, and that hospital staff may acquire strains from outbreaks and excrete them in their feces (Chenoweth and Schaberg, 1990; Johnson, 1994). This also demonstrates that enterococci colonize the gastrointestinal tract. Colonization may not constitute a virulence factor as such, but it may amplify potential pathogenicity of a strain in combination with other virulence factors. However, specificity for particular host tissues and the production of aggregation substance (discussed below) may play a major role in pathogenicity.

1.3.9.2. *Adherence.* Adherence of pathogens to the extracellular matrix of various host tissues is considered crucial for infection. Both specific adhesin-ligand as well as hydrophobic interactions may be involved (Zareba et al., 1997). In this context, both *E. faecium* and *E. faecalis* were shown to bind specific extracellular matrix proteins, especially thrombospondin, lactoferrin and vitronectin; however, the cell surface components responsible for binding were not isolated (Zareba et al., 1997). The role of enterococci in UTI and endocarditis suggests that these bacteria are efficient colonizers of these host tissues. The tendency for bacteria to infect specific tissues is often related to their ability to adhere to the respective target cell *in vitro* (Guzmàn et al., 1989).

Strains of *E. faecalis* isolated from UTI's had a greater capacity to adhere to urinary tract epithelial cells and human embryo kidney cells than *E. faecalis* isolated from cases of endocarditis. Conversely, isolates from cases of endocarditis showed better adherence to Girardi heart cells than to urinary tract epithelial cells or human embryo kidney cells. All isolates from cases of endocarditis, but only some from UTI's, exhibited an *in vitro* potential to be highly invasive, and isolates from cases of endocarditis associated less efficiently with human polymorphonuclear leukocytes than those from UTI (Guzmàn et al., 1989). After growth in serum, strains from UTI and endocarditis adhered more efficiently to both human embryo kidney and Girardi heart cell lines, and associated less efficiently with human polymorphonuclear leukocytes. These findings led the authors to suggest that enterococci causing endocarditis may originate from UTI. More invasive UTI strains could attack the kidneys, causing pyelonephritis, and could cause bacteremia. Persistence in blood would change surface antigen expression, making the cell more resistant to phagocytosis and favoring adherence to cardiac cells (Guzmàn et al., 1989). Persistence in a habitat which favors the selection of bacteria with high adherence ability may render some surface modifications induced by blood factors irreversible; this would explain why isolates from cases of endocarditis adhere better to cardiac cells than UTI isolates (Guzmàn et al., 1989).

Aggregation substance (AS) produced in response to sex pheromones (see above) mediates adhesion of *E. faecalis* to cultured renal tubular cells (Kreft et al., 1992). An Arg-Gly-Asp-Ser amino acid motif in the AS is believed to be involved in binding to

eukaryotic cells. This amino acid motif is also found in fibronectin and it mediates the binding to eukaryotic cells by a class of receptors known as integrins (Kreft et al., 1992). Expression of AS can be induced in serum by an unidentified factor (Kreft et al., 1992), and it was argued that this enables the bacterium to 'sense' the eukaryotic environment and to respond by synthesizing AS adhesin (Wirth, 1994). It was also suggested that aggregation substance plays a role in invasion of cultured cells. *E. faecalis* strains expressing AS were internalized by enterocytes in higher numbers than non-expressing mutant strains (Olmsted et al., 1994). Electron microscopy showed that AS interacted with the surface of the enterocyte microvillus and that intracellular enterococci were localized within membrane-bound vacuoles in the cytoplasm of the enterocyte, clearly demonstrating that AS is an important virulence factor (Olmsted et al., 1994).

1.3.9.3. *Translocation.* A large proportion of enterococcal infections are thought to originate from the intestinal tract. According to the translocation model, intestinal epithelial cells or intraepithelial leukocytes phagocytose bacteria adhering to them at the lumen side. The bacteria exit on the apical side of epithelial cells or migrate in phagocytes to mesenteric lymph nodes, proliferate, and spread to distant sites (Jett et al., 1994). A murine model for enterococcal translocation showed that under appropriate conditions of intestinal overgrowth with antibiotic resistant *E. faecalis*, the bacteria could translocate across an intact epithelium and cause systemic infection (Wells et al., 1990).

1.3.9.4. *Resistance to host defense mechanisms.* Most studies on resistance to host defense mechanisms have concentrated on interaction with polymorphonuclear leukocytes (Johnson, 1994). As mentioned above, Guzmàn (1989) showed that isolates of *E. faecalis* from cases of endocarditis grown in broth adhered to polymorphonuclear leukocytes less readily than isolates from UTI. *E. faecalis* isolates from cases of both endocarditis and UTI that were grown in serum showed decreased adherence to polymorphonuclear leukocytes (Guzmàn et al., 1989). *In vitro* phagocytosis assays with *E. faecium and E. faecalis* showed that efficient killing requires the presence of serum complement, and that antibodies to enterococci enhance polymorphonuclear leukocyte-mediated killing (Harvey et al., 1992; Jett et al., 1994; Johnson, 1994). The enterococci

also express a flavin-containing NADH peroxidase which degrades hydrogen peroxide, and they possess an oxygen-inducible superoxide dismutase which catalyzes conversion of superoxide to hydrogen peroxide. However, whether these enzymes enhance survival in macrophages after phagocytosis requires further investigation (Jett et al., 1994).

The group D antigen of enterococci is a membrane-associated lipoteichoic acid which may bind reversibly to human erythrocytes (Beachey et al., 1979). This may be relevant to inflammation, because lipoteichoic acid bound to eukaryotic cells retains antigenic specificity and such cells can suffer complement-mediated lysis when exposed to plasma (Hummell and Winkelstein, 1986). Thus, tissue damage may occur at sites of infection from complement activation by membrane-associated bacterial lipoteichoic acid of the host cell (Jett et al., 1994). In addition, lipoteichoic acid from several enterococcal species stimulated monokine production from cultured human monocytes (Bhakdi et al., 1991). Lipoteichoic acid is also thought to be the binding substance of pheromone-producing cells which recognizes AS of plasmid-bearing donor cells (see above). Therefore, enterococcal lipoteichoic acid may serve as a virulence factor because it may modulate inflammatory responses and facilitate plasmid transfer (Jett et al., 1994).

1.3.10. *Pathology of enterococcal infection.*

Pathological changes associated with enterococci include acute inflammation (Johnson, 1994). Sex pheromones and surface exclusion proteins are thought to be involved in eliciting an inflammatory response. These compounds are chemotactic for human and rat polymorphonuclear leukocytes *in vitro*, and induce superoxide production and secretion of lysosomal enzymes (Ember and Hugli, 1989; Sannomiya et al., 1990; Johnson, 1994). Another pathological change in bacterial endocarditis involves platelet activation and accumulation, leading to development of endocardial vegetations (Johnson, 1994). *E. faecalis*, *E. faecium* and *E. avium* induce platelet aggregation *in vitro*, with concomitant release of serotonin (Usui et al., 1991; Johnson, 1994). Many, but not all, pathogenic strains of enterococci produce cytolysin, which has been linked to increased virulence in animal models, such as murine peritonitis and rabbit endophthalmitis (Ike et al., 1984; Jett et al., 1992). Cytolysin induces tissue damage, as

shown in the endophthalmitis model (Stevens et al., 1992). In the rabbit model for endocarditis, cytolysin contributed to virulence only when associated with AS (Chow et al., 1993). Batish et al. (1990) observed a close relationship between hemolysin production of enterococci, lethality in mice and dermonecrosis in rabbit skin. In Japan it was shown that 60% of clinical strains involved in parenteral infection showed a hemolytic phenotype, compared with only 17% of isolates from feces of healthy individuals (Ike et al., 1987). Cytolysin is involved in, but is not a prerequisite for virulence because nonhemolytic strains of enterococci may also cause infection (Johnson, 1994). Gelatinase is a protease that hydrolyzes gelatin, collagen, hemoglobin and other bioactive peptides (Coque et al., 1995), and it is also considered to be involved in pathogenicity of *Enterococcus* species. Kühnen et al. (1988) showed that 63.7% of strains of *E. faecalis* isolated from surgical intensive care units in Germany produced protease.

Enterococci may produce hyaluronidase. Because production of this enzyme was linked to pathogenesis of other microorganisms, it was suggested that it may also play a role in enterococcal pathogenesis. However, there is no direct evidence for the role of hyaluronidase in disease caused by enterococci (Jett et al., 1994). Coque et al. (1995) investigated the incidence of hemolysin, gelatinase and AS among enterococci isolated from cases of endocarditis, other infections, and from feces of hospitalized and community-based individuals. Hemolysin production was detected at a higher frequency among isolates from clinical specimens (17 to 60%) compared with isolates from feces of healthy individuals (17%). This was in accordance with information reported by Ike et al. (1987); however, hemolysin production was not a common trait among isolates from endocarditis (16%), hence hemolysin may not be necessary for enterococci to cause this type of infection (Coque et al., 1995). Gelatinase and AS both occurred more frequently among clinical isolates than among fecal isolates from healthy volunteers, indicating that these two factors may also play a role in virulence of enterococci. While most clinical isolates of *E. faecalis* exhibited at least one of the three possible virulence factors, more than 45% of isolates from cases of endocarditis lacked hemolysin, gelatinase or AS, indicating that other properties must be important in the pathogenesis of *E. faecalis* endocarditis (Coque et al., 1995). In addition, none of the non-*E. faecalis* strains (*E. faecium, E. gallinarum, E. casseliflavus, E. raffinosus* and other species) exhibited any of

these 'virulence' traits, suggesting that unknown factors may play a role in pathogenesis (Coque et al., 1995).

1.4. Implications of enterococci in foods.

Enterococci, especially *E. faecium* and *E. faecalis*, may be considered as opportunistic pathogens. Infections usually occur nosocomially in persons who are debilitated, have an underlying disease, or have received medical instrumentation. However, the incidence of infections caused by enterococci, their seriousness, and the increasing difficulty of treating such infections because of multiple antibiotic resistance, place these microorganisms among the most important emerging human pathogens. Sources of enterococci involved in human infection were thought to be from the patient's endogenous microflora; however, person-to-person transmission of enterococci in hospital outbreaks has been reported, as well as stool carriage of strains implicated in outbreaks (Moellering, 1991; Jordens et al., 1994; Gordts et al., 1995). Discussion has focused on whether pathogenic enterococci can be transmitted by foods and cause disease in a hospital setting, particularly with focus on VRE. It was also thought that VRE originated in the hospital environment and that they are disseminated to the community, but several researchers have proposed the opposite (Bates et al., 1993, 1994; Klare et al., 1995a,b; Das et al., 1997). A proposed source of VRE is farm animals in which there has been ergotropic use of avoparcin, a glycopeptide antibiotic (Klare et al., 1995a,b; Das et al., 1997). VRE have been isolated from a wide variety of farm animals, and these constitute an important reservoir of VRE that could be transmitted to the hospital environment via contaminated meat (Klare et al., 1995a,b; Devriese et al., 1996). Chadwick et al. (1996) isolated VRE from chicken, pork and beef samples from retail markets in the U.K. and suggested that *vanA* resistance genes may be introduced into the community via the food chain. VRE were also isolated from raw sewage, farm animals and uncooked chicken by Bates et al. (1994), and, more importantly, they showed that blood and urine isolates from different hospital patients and a porcine isolate shared the same ribotyping pattern. These findings strongly suggest that food transmission occurred and, as a result, two European countries (Denmark and Germany) banned the use of

avoparcin (Morrison et al., 1997), followed by a European Union-wide ban (McDonald et al., 1997). However, in a recent study, Klein et al. (1998) could not establish any direct connection between the occurrence of VRE in minced meat and nosocomial infections.

In the U.S.A. a different situation exists with nosocomial VRE infections, because avoparcin has not been licensed for use (McDonald et al., 1997). A community prevalence survey failed to isolate VRE from healthy volunteers without hospital exposure or from environmental sources or probiotic preparations (Coque et al., 1996). In contrast to Europe, transmission of VRE in the U.S.A. does not appear to be from the community to the hospital, and food has not been implicated as a possible vehicle for transmission. This raises the question of the source of VRE isolates in the U.S.A. McDonald et al. (1997) proposed that undetected community transmission of VRE may occur at low levels. Alternatively, it was proposed that enterococci acquired vancomycin resistance genes from an unknown gastrointestinal bacterium (Rice, 1996; Morrison et al., 1997).

The important question is whether enterococci originating from food and from community sources possess equal pathogenic potential, or whether a difference in pathogenicity exists among enterococci from different sources. To answer this, antibiotic resistance patterns and other virulence factors of enterococci isolated from community and clinical sources should be compared. Valdivia et al. (1996) showed that the incidence of antibiotic resistance, as well as aggregation response to sex pheromones, was much higher in clinical strains than isolates from municipal waste-water. It has been reported that strains of enterococci isolated from dairy products do not produce hemolysin (Arihara et al., 1993; Giraffa, 1995), and it was suggested that absence of hemolytic activity should be a selection criterion for starter strains for dairy use (Giraffa, 1995). It is now known that hemolytic activity is not necessarily associated with all clinical isolates (see above); therefore, absence of hemolytic activity in enterococci isolated from food does not mean that these bacteria are nonvirulent. Enterococci from food should be investigated for presence of other potential virulence factors, such as AS or protease.

Antibiotic resistant enterococci have been isolated from foods such as raw milk cheeses, raw meats and sausages (Batish and Ranganathan, 1986; Knudtson and Hartman, 1993b; Perreten and Teuber, 1995; Perreten et al., 1997; Wegener, et al., 1997). For example,

Teuber et al. (1996) showed that enterococci isolated from Salami and Landjäger-types of fermented sausage were frequently resistant to streptomycin and lincomycin, while isolates from Emmental and Appenzeller cheeses showed a high frequency of resistance to erythromycin, gentamicin, tetracycline and/or vancomycin. Enterococci harboring conjugative antibiotic resistance transposons and (or) plasmids are present in the human food chain and antibiotic resistance was transferred by filter matings to foodborne pathogens, such as *L. monocytogenes* and *S. aureus*, as well as to other bacteria which are commonly associated with foods, such as *L. lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides* (Perreten et al., 1997). There have not been any comprehensive studies on possible differences in antibiotic resistance patterns and virulence factors of food and clinical isolates. However, molecular characterization of some resistance determinants, showed them to be similar to those from clinical specimens. These observations also included resistance transfer mechanisms such as conjugative plasmids and transposons (Teuber et al., 1996; Perreten et al., 1997).

The principal concern for enterococci in the food supply is their pathogenic potential based on horizontal transfer of genes of factors associated with virulence and antibiotic resistance. In the hospital setting, enterococci acquire multiple antibiotic resistance from their efficient gene transfer mechanisms, which may also encode factors which are associated with virulence, i.e., aggregation substance, hemolysin or gelatinase (Coque et al., 1995). Whether or not these bacteria should be considered pathogens in food, the concern is that in the hospital setting they can rapidly acquire plasmid-encoded genes for antibiotic resistance and virulence traits, and can become pathogenic. The enterococci used in foods or as probiotics are usually strains of *E. faecium*. Most bacteriocin-producing enterococci described for use as starter cultures are also strains of *E. faecium*. The pathogenic potential of *E. faecalis* is considered to be greater than that of *E. faecium*, because greater than 80% of enterococci associated with human infections are *E. faecalis* (Jett et al., 1994). This may reflect the fact that transfer of plasmids in reponse to sex pheromones has only been described for *E. faecalis*. This genetic transfer mechanism is highly efficient and it is thought to be associated with virulence factors. On the other hand, vancomycin-resistance has been linked to strains of *E. faecium* (Gordts et

al., 1995; Morrison et al., 1997), and the importance of vancomycin-resistant *E. faecium* in nosocomial disease cannot be disregarded.

Even though *E. faecalis* seems to have a greater pathogenic potential than *E. faecium*, the association of either of these species with food may not be considered desirable. Although present evidence does not suggest that enterococci should be regarded as foodborne pathogens, the food chain has clearly been established as an important source of enterococci in the human environment, some strains of which - albeit at a low frequency - may bear resistance traits to glycopeptide and other antibiotics. However, the incidence of enterococci in human disease does not appear to correlate with the incidence of these organisms in foods, especially when their use as starter cultures or as probiotics is taken into consideration. On the other hand, Teuber et al. (1996) concluded from data obtained over 15 years that fermented foods such as cheese and cured sausages may contribute to the distribution of antibiotic-resistant bacteria (staphylococci and enterococci) to the consumer. With foods as a potential source of enterococci bearing plasmids for antibiotic resistance and factors associated with virulence, it would be prudent for food manufacturers to be cognizant of these organisms and to exercise better control over their presence in foods than is presently the case.

The implications of food transmission and intestinal colonization of enterococci for their control in the food supply are not clear. On the positive side, enterococci are associated with desired flavor attributes of some Southern European cheeses, whilst in some probiotics they represent the dominant or sole organism. However, in most cases the enterococci constitute an unnecessary adventitious microflora of foods, especially of heat-treated and processed products. For example, enterococci should not be considered an acceptable microflora of in-package heated meats. While reworking of processed meat in an industrial setting makes economic sense, it may lead to the establishment of enterococci as the predominant microflora of the meat. These bacteria are not desired because of their potential role in spoilage and quality defects. Moreover, if they carry antibiotic resistance genes and possible virulence factors, they would constitute a health risk. Special care should be taken that enterococcal strains used as probiotics or starter cultures, do not acquire antibiotic resistance and that they do not carry potential virulence factors. Such a risk may especially arise when 'back-slopping' techniques are applied.

Of further concern is the potential risk of vertical transfer of antibiotic resistance and virulence traits to other LAB in foods. This is especially true if antibiotic resistance genes or possible virulence traits are encoded on broad-host-range plasmids. Although streptococci and enterococci are the predominating LAB associated with human infections, other LAB have been implicated in human infections, albeit at a low incidence and mainly associated with immunodeficient hosts (Aguirre and Collins, 1993; Gasser, 1994).

Clearly, the safety of foods that contain enterococci is an issue that the food industry must address. Control of enterococci in foods is important especially for those consumers who are at highest risk, i.e., the elderly, the hospitalized and the immunocompromized. The food supply should be safe for all, especially for those who are at risk of opportunistic infections. The control of these organisms constitutes a special challenge because of their robust nature, their wide distribution and their stability in the extraenteral environment. In foods where other LAB grow as a competitive microflora, the use of bacteriocinogenic LAB strains with specific activity against enterococci, offers a strategy for their control. Enterocins active against a wide range of enterococci other than the producer strain show potential for such control. The genes necessary for enterocin production may be transferred to other LAB used as starter cultures and which are generally recognized as safe (GRAS). In this way, growth of enterococci could be inhibited by the enterocin produced by a heterologous host, and transmission of potentially pathogenic enterococci in a food would be prevented.

1.5. Research objective

At the initiation of this study bacteriocin production by most LAB genera had been described; however detailed studies on the genetics and chemistry of bacteriocins were generally limited to class II bacteriocins produced by the genera *Lactococcus, Leuconostoc, Lactobacillus* and *Pediococcus.* Relatively little was known about bacteriocins of the genus *Enterococcus,* apart from the fact that production of bacteriocins among enterococci had been reported and that antimicrobial activity against *Listeria monocytogenes, Clostridium* spp. and other enterococcal strains appeared to be a common trait. Therefore, the objective of this study was to obtain an *Enterococcus* sp. from food which produced a bacteriocin active against other enterococci as well as foodborne pathogens such as *Listeria monocytogenes,* and to investigate the possibility of utilizing the genes responsible for bacteriocin production for heterologous expression in suitable LAB starter cultures used in biopreservation of meats. To reach this objective the study aimed to:

1. Isolate and identify a bacteriocin producing *Enterococcus* sp. from food.

2. Characterize the bacteriocin with respect to its spectrum of activity, heat stability, and conditions for production.

3. Purify the bacteriocin to homogeneity and to determine it's molecular mass.

4. Clone the genetic determinants associated with bacteriocin production and immunity by reverse genetics.

5. Express the genetic determinants for bacteriocin production and immunity in a heterologous host.

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CHAPTER 2*

ISOLATION AND IDENTIFICATION OF A BACTERIOCIN-PRODUCING *Enterococcus faecium* FROM BLACK OLIVES, AND CHARACTERIZATION OF ITS BACTERIOCIN.

2.1. Introduction.

Bacteriocin production has been observed among most LAB genera, i.e., enterococci, carnobacteria, lactobacilli, lactococci,, leuconostocs, and pediococci (Klaenhammer, 1993; DeVuyst and Vandamme, 1994). Generally, these bacteriocins are active against bacteria closely related to the producer strain, but many are also active against foodborne pathogens such as *L. monocytogenes, B. cereus, C. perfringens* and *S. aureus,* and are therefore considered to be natural food preservatives (Abee et al., 1995; Holzapfel et al., 1995; Schillinger et al., 1996; Stiles, 1996). Bacteriocins from LAB can be divided into two groups on the basis of their structure. The first group contains the lantibiotics which consist of a polypeptide chain that has been posttranslationally modified. The modified amino acids are lanthionine and β-methyllanthionine and their precursors dehydroalanine and dehydrobutyrine (Klaenhammer, 1993, Sahl et al, 1995). The second group are nonlantibiotics and consist of small (less than 10-kDa), heat stable, cationic and hydrophobic peptides containing only unmodified amino acids (Klaenhammer, 1993; Jack et al., 1995). Both lantibiotics and nonlantibiotics are ribosomally synthesized as precursor peptides containing an N-terminal leader sequence which is cleaved concomitant with export of bacteriocin from the cell (Håvarstein et al., 1995; Nes et al., 1996).

LAB bacteriocins were also divided into three major classes by Klaenhammer, (1993) and Nes et al. (1996). Class I includes the lantibiotics. The most extensively

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studied food-related bacteriocin of this class is nisin, which is used world-wide to preserve foods such as canned foods and processed cheeses (Delves-Broughton, 1990). Class II bacteriocins are the small, heat stable nonlantibiotics that do not undergo posttranslational modification except for, in some cases, the formation of disulfide bridges. Class III bacteriocins consist of large, heat labile proteins. A fourth class of bacteriocins was proposed to include those that form complexes with lipids or carbohydrates, but these are not well characterized and it is uncertain whether the additional nonprotein chemical moieties are essential for their activity (Klaenhammer, 1993; Nes et al., 1996). The classification of class II bacteriocins was recently modified by Nes et al. (1996): Class IIa comprises the pediocin-like bacteriocins which generally have a strong antilisterial effect; class IIb includes the two peptide bacteriocins; and class IIc consists of the *sec*-dependant secreted bacteriocins.

The production of bacteriocins *in situ* by 'protective' cultures has been studied in several food and model food systems. The majority of these inoculation experiments were performed with meats and meat products, but fish, dairy and vegetable products have also been used (Holzapfel et al., 1995; Schillinger et al., 1996; Stiles, 1996). These protective cultures were used with the intention of demonstrating the effects of the bacteriocin on inhibition of a target pathogen such as *L. monocytogenes* (Muriana, 1996; Schillinger et al., 1996; Stiles, 1996), or they were used in preservation studies to demonstrate an increase in product shelf life (McMullen and Stiles, 1996; Schillinger et al., 1996; Stiles, 1996). Probably the majority of bacteriocin-producing LAB have been isolated from dairy or meat products. Some bacteriocin-producing isolates have also been obtained from vegetable products, for example a *L. plantarum* strain that dominated in the fermentation of Spanish green olives was found to be bacteriocinogenic (Ruiz-Barba et al., 1994). Bacteriocin-producing *L. lactis* strains were isolated from vegetables, (Uhlman et al., 1992; Franz et al., 1997) and a bacteriocinogenic *L. plantarum* strain from a ready-to-eat 'Waldorf' salad (Franz et al., 1998).

This study was initiated at the Federal Research Centre for Nutrition, Institute of Hygiene and Toxicology in Karlsruhe, Germany. This institute has a strong research program concerned with food hygiene and specific projects deal with application of bacteriocin-producing protective cultures to improve the hygiene of vegetable-type foods.

The objective of this study was to isolate and identify new bacteriocin-producing LAB of vegetable origin for use as protective cultures, and to characterize their bacteriocins with respect to their activity spectrum, production kinetics, bactericidal activity, proteinaceous nature and heat stability. Bacteriocin-production by *L. lactis* and *L. plantarum* strains isolated in the course of this study was reported previously (Franz et al., 1997, 1998). This chapter describes bacteriocin production by an *Enterococcus faecium* strain isolated from black olives.

2.2. Methods and Materials.

2.2.1. *Isolation of lactic acid bacteria.*

LAB were isolated from a variety of vegetable products including raw spinach, carrots, broccoli, sweet potato and bean sprouts and fermented vegetables including: sauerkraut, black olives and green beans. Twenty gram samples were weighed into stomacher bags which were sealed and incubated at 15°C for three days to boost LAB numbers. Isolates were obtained by homogenizing the 20 g samples in 180 ml quarter-strength Ringers solution (Merck, Darmstadt, Germany), and plating 0.1 ml of suitable tenfold dilutions in quarter-strength Ringers solution onto modified MRS agar (Merck, Darmstadt, Germany) with 0.1% cysteine monohydrochloride and 0.2% potassium sorbate added, pH 5.7. Predominant colony types were picked off the modified MRS agar plates and purified by streaking out onto MRS agar.

2.2.2. *Screening for bacteriocin-producing LAB isolates.*

All gram-positive, catalase negative isolates were identified as presumptive LAB and screened for antagonistic activity by the deferred inhibition method (Ahn and Stiles, 1990) using the bacteriocin screening medium of Tichaczek et al. (1992). Isolates that showed antagonistic activity against at least one of four indicator strains used (*L. sake*, DSM 20017, *L. mesenteroides* DSM 20343, *L. plantarum* DSM 20174 and *E. faecalis* DSM 20380) were kept for identification and further characterization of the bacteriocin.

2.2.3. *Cultures and media.*

LAB were grown aerobically at 30°C in MRS broth. *Bacillus, Staphylococcus* and *Listeria* strains were grown aerobically at 30°C in Standard One Broth (Merck, Darmstadt, Germany). *Clostridium* strains were grown anaerobically in Reinforced Clostridial Medium (Unipath, Wesel, Germany) at 37°C. Stock cultures were kept in the media used to grow cells with 15% glycerol added and stored at -80°C.

2.2.4. *Identification of bacteriocin-producing isolates.*

Growth of isolates at pH 9.6, 6.5% sodium chloride and at temperatures of 6, 10, 15 and 45°C was determined in MRS broth. Gas (CO_2) production from glucose, arginine hydrolysis, fermentation of carbohydrates, the configuration of lactic acid enantiomers produced and the presence of meso-diaminopimelic acid in cell walls were determined by the methods of Schillinger and Lücke (1987). In addition, isolates were identified by comparing their total soluble cell protein profile to that of reference strains by SDS-PAGE. For this comparison, LAB were grown in MRS broth for 24 h at 30°C. Total soluble protein was isolated by centrifugation of 1 ml of culture in a microcentrifuge and washing cells once with phosphate buffer (10 mM sodium phosphate buffer containing 0.8% sodium chloride, pH 7.3). Cells were resuspended in 150 µl of lysis solution (25 mM Tris pH 7.5, 10 mM EDTA pH 8.0, 0.9% glucose and 10 mg/ml lysozyme) and held at 30°C for 1 h. The cell extract was centrifuged in a microcentrifuge and the pellet was resuspended in 100 µl SDS loading buffer (0.75 g Tris, 5 ml Mercaptoethanol, 10 g glycerol, 2% SDS and 0.1% Bromophenol blue per 100 ml; pH 6.8). Protein samples were boiled for five min and cooled on ice. Samples were subjected to SDS-PAGE according to Pot et al. (1994). Gels were dried using a Biorad oven and scanned on a flatbed scanner. Registration of the protein electrophoretic pattern, normalisation of the densitometric traces, grouping of strains by the Pearson product moment correlation coefficient (r) and unweighted pair group method using arithmetic averages (UPGMA) cluster analysis was performed by techniques described by Pot et al. (1994) using the software package GelCompar, version 2.0 (Vauterin and Vauterin, 1992).

Homology between DNA of isolates and reference strains was determined. DNA of LAB strains was isolated and purified according to the method of Marmur (1961) modified as described by Stackebrandt and Kandler (1979). The DNA base composition was estimated from the thermal melting point of DNA as described by Marmur and Doty (1961), using a Gilford Response spectrophotometer. The spectrophometric determination of DNA-DNA hybridization from renaturation rates was performed using the modified (Huss et al., 1983) optical method of DeLey et al. (1970).

2.2.5. *Partial purification, activity assay and spectrum of activity.*

The antagonistic compound produced by a bacteriocin-producing *E. faecium* BFE 900 strain was partially purified. The producer strain was grown aerobically in 1 liter of MRS broth (pH 7.0) for 18 h at 30°C without pH control. After growth, the culture was heated at 70°C for 30 min, and centrifuged at 11,700 x *g* for 40 min. Culture supernatant (1 liter) was loaded onto an Amberlite XAD-8 (BDH Chemicals Ltd., Poole, UK) hydrophobic interaction column (150 x 75 mm). The column was washed with 1.5 liter of 0.1% trifluoroacetic acid (TFA) followed by 1 liter of 20% ethanol in 0.1% TFA. The antimicrobially active fraction was eluted with 1 liter of 40% ethanol in 0.1% TFA, and concentrated to 10 ml by rotary evaporation. This fraction was adjusted to pH 6.5 with 6 N sodium hydroxide and sterilized by filtration through a 0.22 µm membrane.

For activity assays, cell-free, neutralized culture supernatant was prepared as follows: an overnight culture of the bacteriocin-producing strain was centrifuged at maximum speed in a microcentrifuge (Eppendorf) for 10 min and the supernatant was adjusted to pH 6.5 to 7.0 with 1 N sodium hydroxide. The neutralized supernatant was boiled for 5 min to inactivate remaining cells. Neutralized supernatant was used in activity assays by the critical dilution method (Schillinger et al., 1993). Overnight indicator cultures were used to seed (1% inoculum) soft agar. Neutralized supernatant was diluted in a doubling dilution series with MRS broth as diluent, and 10 µl volumes of suitable dilutions were spotted onto indicator plates. One arbitrary activity unit (AU) was defined as the reciprocal of the highest dilution yielding a clear zone of inhibition on the

indicator lawn, and was multiplied by a factor of 100 to obtain the AU ml^{-1} of the original sample.

The cell free, neutralized supernatant (6,400 AU ml^{-1} activity against *L. sake* DSM 20017) was tested for activity against a wide range of LAB and non-LAB indicator strains (listed in Table 2.2 in results section). For comparison, the partially purified fraction (51,200 AU ml^{-1} activity against *L. sake* DSM 20017) was also tested for activity against the same indicators.

2.2.6. *Effect of heat, enzymes and pH on bacteriocin activity.*

Cell free, neutralized supernatant (6,400 AU ml^{-1}) was boiled for 5, 10, and 20 min and was autoclaved at 121°C for 15 min. The neutralized supernatant was also treated with the following enzymes at a final concentration of 1 mg ml^{-1}: α-chymotrypsin (pH 7.0; Serva, Heidelberg, Germany), trypsin (pH 7.0; Sigma, Deisenhofen, Germany), pepsin (pH 3.0; Merck, Darmstadt, Germany), proteinase K (pH 7.0; Sigma), α-amylase (pH 7.0; Sigma), lipase (pH 7.0; Sigma), catalase (pH 7.0; Sigma), and lysozyme (pH 7.0; Serva). Enzyme activity was inactivated after 2 h by boiling for 5 min. Untreated samples were used as controls. In addition, neutralized supernatants of the known bacteriocin-producing cultures *L. sake* Lb706 and *L. carnosum* LA54A were included to confirm enzyme activity when applicable. After heat or enzyme treatment, remaining bacteriocin activity was assayed using *L. sake* DSM 20017 as the indicator strain.

2.2.7. *Bactericidal activity.*

Inactivation of a population of sensitive *L. sake* DSM 20017 cells by neutralized supernatant of an overnight *E. faecium* BFE 900 culture was studied by adding 1 ml of the supernatant (6,400 AU ml^{-1}) to 8 ml of MRS broth. A *L. sake* indicator culture in the stationary phase of growth (grown 24 h in MRS broth at 30°C) was diluted in MRS broth, and 1 ml of a suitable dilution was inoculated into bacteriocin-containing MRS broth to a final concentration of *ca.* 10^6 CFU ml^{-1}. As a control, 9 ml of MRS broth containing no bacteriocin was inoculated with 1 ml of the same diluted indicator culture. Cultures were

incubated at 30°C and CFU ml^{-1} of *L. sake* cells was determined at selected time intervals by spread plating 0.1 ml of suitable tenfold dilutions on MRS agar.

2.2.8. *Kinetics of bacteriocin production.*

MRS broth (60 ml, pH 7.0) was inoculated with *E. faecium* BFE 900 at a level of 5×10^4 CFU ml^{-1}. Viable counts (CFU ml^{-1}) and bacteriocin activity (AU ml^{-1}) in the culture supernatant were determined at the time of inoculation (0 h) and at 2-h intervals during aerobic incubation at 30°C.

2.2.9. *Effect of pH on bacteriocin activity.*

To determine the effect of pH on the activity of the *E. faecium* BFE 900 bacteriocin, cell-free supernatant (6,400 AU ml^{-1}) was adjusted to pH levels between pH 2 and 10 using 6 N sodium hydroxide or hydrochloric acid. Samples were assayed for activity after 24 h at 4°C by the critical dilution method with *L. sake* DSM 20017 as indicator strain. To correct for inhibition due to pH, samples treated with proteinase K before adjusting pH were tested against the same indicator.

2.2.10. *Influence of medium pH and components on bacteriocin production.*

MRS broth adjusted to pH 3, 4, 5, 6, 7, 8, 9, and 10 was inoculated (1%) with bacteriocin-producing culture (*ca.* 10^7 CFU ml^{-1}) grown overnight at 30°C. To determine the effect of medium components on bacteriocin production, different MRS-based media formulations (Table 2.1) and the CAA medium of Hastings et al. (1991) were adjusted to pH 6.5 and also inoculated (1%) with bacteriocinogenic culture grown at 30°C overnight. For both the pH and the medium component studies the cultures were incubated aerobically at 30°C for 24 h, after which the number of CFU ml^{-1} and bacteriocin activity (AU ml^{-1}) in the culture supernatant were determined.

Table 2.1 Media used to determine effect of medium components on bacteriocin production by *Enterococcus faecium* BFE 900.

Medium	Medium components (g l^{-1})			
	Peptone	Yeast extract	Meat extract	Tween 80
M1	10	5	2	1
M2	10	5	2	0
M3	10	5	0	0
M4	10	0	0	0
M5	5	5	0	0
M6	5	2.5	0	0
M7	2.5	7.5	0	0
M8	2.5	5	0	0
M9	2.5	2.5	0	0
M10	0	5	0	0
CAA[a]	15[b]	5	0	1

[a] Hastings et al. (1991), [b] peptone replaced by casamino acids (Hastings et al., 1991)

2.2.11. *Plasmid DNA isolation.*

Plasmid DNA was isolated on a small scale according to the methods of Keppler et al. (1991) and O'Sullivan and Klaenhammer (1993). The known bacteriocin producer *L. carnosum* LA54A was used as a positive control, because this bacterium contains a plasmid of *ca.* 60 kb (Keppler et al., 1991). DNA samples were subjected to electrophoresis on horizontal 0.7% agarose gels in 0.5 x Tris Borate EDTA (TBE) buffer (O'Sullivan and Klaenhammer, 1993).

2.3. Results.

2.3.1. *Identification of bacteriocin-producing LAB.*

Eleven out of 408 presumptive LAB showed antagonistic activity against one or more of the indicator strains tested. Strain BFE 900 isolated from fermented black olives exhibited antagonistic activity against most of the indicator strains (Table 2.2) when the neutralized supernatant was tested and this strain was selected for further study. Cells of this strain were coccoid in shape as determined by phase contrast microscopy. The isolate grew at 10 and 45°C, as well as in the presence of 6.5% sodium chloride. Cells were able to hydrolyze arginine and did not contain meso-diaminopimelic acid in the cell wall. The culture's inability to produce gas from glucose, the formation of only L (+)-lactic acid, its ability to grow at pH 9.6 and 45°C and the sugar fermentation pattern allowed its preliminary identification as *Enterococcus faecium.*

Analysis of total soluble protein patterns by SDS-PAGE confirmed the identity of the isolate as *E. faecium.* The isolate formed a cluster with the *E. faecium* DSM 20477 and *E. faecalis* DSM 20478 reference strains at r = 82.1% (Fig. 2.1). The isolate was more closely related to the *E. faecium* DSM 20477 reference strain (r = 89.5%) than to the *E. faecalis* DSM 20478 reference strain r = 82.1%. Together the enterococci formed a cluster with the more distantly related *Lactococcus* isolates (BFE 901, 902, 903, 904 and BFE 920, Franz et al., 1997) and reference strains (DSM 20729 and 20384) at r = 72.8% (Fig. 2.1). In DNA-DNA hybridization studies, isolate BFE 900 showed 100% homology to the *E. faecium* DSM 20477 reference strain, but only 24% homology to the *E. faecalis* DSM 20478 negative control. This also confirmed the identity of the isolate as *E. faecium.*

Table 2.2 Antagonistic activity of neutralized supernatant and partially purified Amberlite XAD-8 fraction from *E. faecium* BFE 900 against lactic acid and non-lactic acid bacteria. Values obtained from duplicate determinations.

Indicator strain	Source[a]	Activity of neutralized supernatant (AU ml^{-1})	Activity of partially purified fraction (AU ml^{-1})
Enterococcus faecalis 20380	DSM	100	400
Enterococcus faecalis 20478	DSM	1600	6400
Enterococcus faecium 20477	DSM	1600	3200
Leuconostoc carnosum 5576	DSM	0	100
Lactobacillus plantarum 20174	DSM	100	800
Lactobacillus plantarum 20205	DSM	0	200
Lactobacillus sake 20017	DSM	6400	51200
Pediococcus acidilactici 20333	DSM	100	400
Bacillus cereus 2301	DSM	0	0
Bacillus cereus 345	DSM	0	0
Clostridium butyricum 552	DSM	100	800
Clostridium difficile 1296	DSM	0	100
Clostridium perfringens 756	DSM	100	800
Listeria innocua 2257	WS	800	3200
Listeria monocytogenes 125	WS	1600	3200
Listeria monocytogenes 2247	WS	800	6400
Listeria monocytogenes 2250	WS	800	6400
Listeria welshimeri 2254	WS	1600	6400
Staphylococcus aureus 14458	ATCC	0	0
Staphylococcus aureus 6538	ATCC	100	400

[a] DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany,
WS, Technical University München-Weihenstephan, Germany,
ATCC, American Type Culture Collection, USA.

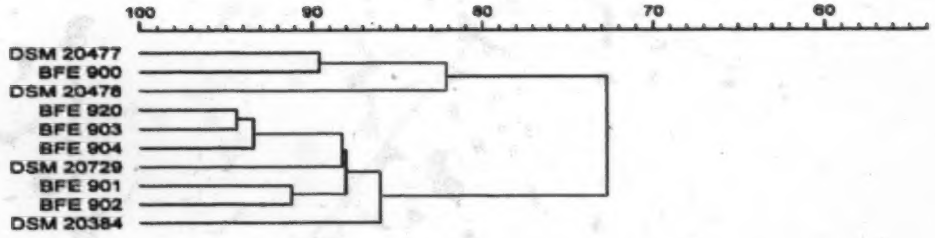

Figure 2.1 Clustering analysis based on protein patterns of enterococci reference strains (DSM 20477 and 20478) and isolate BFE 900, as well as *Lactococcus lactis* isolates (BFE 901, 902, 903, 904, 920; Franz et al., 1997) and reference strains (DSM 20729 and 20384).

2.3.2. *Effect of enzymes on the antibacterial activity of neutralized supernatant.*

Antagonistic activity of neutralized supernatant from *E. faecium* BFE 900 could be inactivated by treatment with proteolytic enzymes (Table 2.3). After treating neutralized supernatant with α-chymotrypsin, trypsin, pepsin and proteinase K, activity decreased from 6,400 AU ml^{-1} to 0 AU ml^{-1}. Antagonistic activity could not be inhibited by catalase, and neutralized supernatant was also not inactivated by lysozyme, α-amylase and lipase (Table 2.3).

2.3.3. *Bactericidal activity of neutralized supernatant.*

Addition of neutralized supernatant to the *L. sake* DSM 20017 indicator culture resulted in a decrease in numbers of indicator cells from an initial log 6.0 CFU ml^{-1} to log 4.0 CFU ml^{-1} after 1 h of incubation at 30°C (Table 2.4). Numbers of indicator cells decreased to *ca.* log 2.0 CFU ml^{-1} after 2 h of incubation, and remained at that level

Table 2.3 Effects of enzymes, heat and pH on inhibitory activity of cell free supernatant of *Enterococcus faecium* BFE 900. Values obtained from duplicate determinations.

Treatment	Activity (AU ml^{-1})
Untreated	6,400
Enzymes:	
α-chymotrypsin (Serva, no. 17160)	0
Trypsin (Sigma, no. T-8253)	0
Pepsin (Merck, no. 7189)	0
Proteinase K (Sigma, no. P-0390)	0
α-amylase (Sigma, type VIIA)	6,400
Lysozyme (Serva, no. 28262)	6,400
Catalase (Sigma, no. C-10)	6,400
Lipase (Sigma, no. L-1754)	6,400
Heat:	
3 min boiling	6,400
10 min boiling	6,400
20 min boiling	6,400
15 min at 121°C	800
pH:	
2	1,600
3	3,200
4	3,200
5	3,200
6	6,400
7	3,200
8	3,200
9	3,200
10	800

Table 2.4 Bactericidal effect of enterocin 900 on *Lactobacillus sake* DSM 20017 in MRS broth at 30°C.

Time of incubation (h)	Viable count (log CFU ml^{-1})	
	Control (no bacteriocin added)	MRS broth with bacteriocin (640 AU ml^{-1})
0	5.9	5.9
1	6.1	4.0
2	6.1	2.3
4	6.8	1.7
7	7.7	2.0
10	8.5	2.5

throughout the remainder of incubation at 30°C. However, viable counts increased from log 6.0 CFU ml^{-1} to log 8.5 CFU ml^{-1} after 10 h at 30°C in MRS broth without added bacteriocin (Table 2.4). Based on the proteinaceous nature of the antimicrobial compound produced by *E. faecium* BFE 900 and its bactericidal mode of action it satisfied the criteria for bacteriocins and it was termed enterocin 900.

2.3.4. *Effect of heat and pH on bacteriocin activity.*

Heating of the neutralized supernatant for 3, 10 and 20 min at 100°C did not affect bacteriocin activity compared with an unheated control. Bacteriocin activity also remained after heating at 121°C for 15 min, but decreased from 6,400 to 800 AU ml^{-1} (Table 2.3). Cell free *E. faecium* BFE 900 supernatant was most active (6,400 AU ml^{-1}) at pH 6.0 (Table 2.3). Bacteriocin activity of the supernatant decreased at pH levels above and below this value. Between pH 3.0 and pH 9.0, however, the bacteriocin activity in the supernatants was still comparatively high (3,200 AU ml^{-1}). At pH 2.0 and pH 10, activity of the supernatants decreased further, but activity could still be detected (Table 2.3).

2.3.5. *Spectrum of activity.*

The activity spectrum of neutralized *E. faecium* BFE 900 culture supernatant and the partially purified fraction is shown in Table 2.2. *Pediococcus acidilactici*, enterococci and two *Lactobacillus* strains were inhibited by the neutralized supernatant; in addition, all of the strains of *L. monocytogenes* and one strain each of *L. innocua, L. welshimeri, C. perfringens, C. butyricum* and *S. aureus* were inhibited. With the partially purified fraction a slightly broader range of activity was observed, including: another strain of *Lactobacillus* as well as a strain of *L. carnosum* and *C. difficile*. Generally, the activity of the partially purified fraction was greater than that of neutralized supernatant (Table 2.2).

2.3.6. *Bacteriocin production kinetics.*

Viable counts and bacteriocin production (AU ml^{-1}) during growth of *E. faecium* BFE 900 in MRS broth (pH 7.0) at 30°C are shown in Fig. 2.2. Counts increased from an initial log 4.0 ml^{-1} to > log 8.0 ml^{-1} during the first 12 h of incubation. The counts then stabilized at *ca.* log 9.0 ml^{-1} and remained at this level until the end of the 132-h incubation period. Bacteriocin production was first detected 8 h after inoculation, when culture density had reached log 8.0 CFU ml^{-1}. Bacteriocin production then increased, reaching a maximum of 3,200 AU ml^{-1} after 12 h of incubation. Bacteriocin activity remained stable at this level for the next 40 h, before declining to a level of 800 AU ml^{-1} after 132 h incubation (Fig. 2.2).

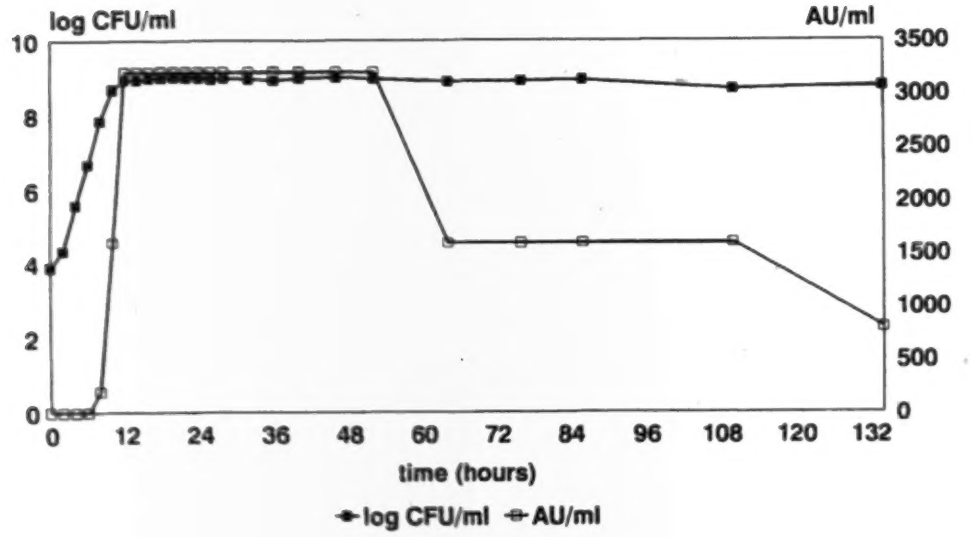

Figure 2.2 Viable count (log CFU ml^{-1}) and bacteriocin production (AU ml^{-1}) of *Enterococcus faecium* BFE 900 during growth in MRS broth (pH 7.0) at 30°C.

2.3.7. *Influence of medium pH and components on bacteriocin production.*

The effect of medium pH on production of bacteriocin is shown in Table 2.5. *E. faecium* BFE 900 grew to a level of log 9.0 CFU ml^{-1} in MRS medium with initial pH 6.0 to 10.0, and produced bacteriocin at a level of 3,200 to 6,400 AU ml^{-1} at these pH values. Growth was slightly reduced at pH values below 6.0, but counts were still higher than log 6.0 CFU ml^{-1}. *E. faecium* BFE 900 did not grow at pH 3.0. Although growth of *E. faecium* BFE 900 occurred at pH 4.0 and 5.0, bacteriocin was not produced within the 24-h incubation period (Table 2.5). Enterocin 900 production was highest at pH 6.0 to 9.0. At pH 10.0 bacteriocin production was reduced.

Table 2.5 Influence of initial pH of MRS medium on numbers (log CFU ml^{-1}) and bacteriocin production (AU ml^{-1}) of *Enterococcus faecium* BFE 900, determined after growth for 24 h at 30°C. Values obtained from duplicate determinations.

pH	Viable count (log CFU ml^{-1})	Activity (AU ml^{-1})
3	-[a]	0
4	6.9	0
5	8.1	0
6	8.8	6,400
7	8.8	6,400
8	9.1	6,400
9	9.3	6,400
10	9.3	3,200

[a] no growth

The effect of medium composition on bacteriocin production is shown in Table 2.6. Although the microorganism grew to comparable levels (*ca.* log 9.0 CFU ml^{-1}) in CAA medium and all MRS-based broth media, bacteriocin production was clearly dependent on the components of the medium. Bacteriocin production was highest in MRS medium (commercial formulation, Merck, Darmstadt, Germany) containing all of the components of the medium tested at their normal concentrations (medium M1, Table 2.6). Omitting Tween 80 from the MRS medium formulation decreased bacteriocin production from 3,200 AU ml^{-1} to 1,600 AU ml^{-1}. Growing the producer strain in MRS medium containing only peptone (M4) or yeast extract (M10) lowered bacteriocin activity from 1,600 to 400 and 200 AU ml^{-1}, respectively. Similarly, when peptone concentration was reduced to a quarter of that of commercial MRS formulation (M8 and M9), bacteriocin production decreased to 400 and 200 AU ml^{-1}, respectively. When the yeast extract concentration in MRS-based medium was increased (M7), bacteriocin was

Table 2.6 Influence of medium composition [CAA medium of Hastings et al. (1991) and modified MRS media M1-M10] on numbers (log CFU ml^{-1}) and bacteriocins production (AU ml^{-1}) of *Enterococcus faecium* BFE 900, determined after growth at 30°C for 24 hours. Values obtained from duplicate determinations.

Medium	Medium components ($g\ l^{-1}$)				Viable count (log CFU ml^{-1})	Bacteriocin activity (AU ml^{-1})
	Peptone	Yeast extract	Meat extract	Tween 80		
M1	10	5	2	1	8.8	3200
M2	10	5	2	0	8.8	1600
M3	10	5	0	0	8.6	1600
M4	10	0	0	0	8.8	400
M5	5	5	0	0	8.7	1600
M6	5	2.5	0	0	8.7	1600
M7	2.5	7.5	0	0	8.8	1600
M8	2.5	5	0	0	8.8	400
M9	2.5	2.5	0	0	8.8	200
M10	0	5	0	0	8.8	200
CAA	15*	5	0	1	8.7	400

detected at 1,600 AU ml^{-1}, even though the peptone concentration was reduced to one quarter of that of commercial MRS. Bacteriocin production occurred to a similar level (1,600 AU ml^{-1}) in MRS-based media in which peptone concentration was halved, while that of yeast extract remained the same or was reduced to half of that of commercial MRS (M5 and M6, respectively, Table 2.6).

2.3.8. Plasmid isolation.

No plasmid DNA could be isolated from *E. faecium* BFE 900 by the two small-scale isolation methods used in this study. Plasmid DNA was readily isolated from the *L. carnosum* LA54 control strain (results not shown), indicating that the methods used in this study were suitable for isolation of plasmid DNA.

2.4. Discussion.

The bacteriocin-producing strain BFE 900 isolated from fermented black olives was identified as *Enterococcus faecium* using 'traditional' identification techniques (biochemical and physiological tests). Enterococci may be distinguished from other gram-positive, catalase-negative cocci by their ability to grow at 10 and 45°C, in 6.5% sodium chloride and at pH 9.6 (Hardie and Whiley, 1997; Morrison et al., 1997). Because strains of other gram-positive, catalase-negative cocci (e.g., leuconostocs, lactococci or streptococci) may exhibit the same properties, these physiological tests do not unequivocally distinguish between these genera (Devriese et al., 1993). For this reason the more modern and accurate identification techniques of DNA-DNA hybridization and SDS-PAGE of total soluble cell proteins were employed for the identification of the bacteriocin-producing isolate. SDS-PAGE of total soluble cell protein distinguished this strain together with the reference strains of *Enterococcus* from *Lactococcus lactis* strains and grouped them as a separate cluster. This method was also successful in identifying the BFE 900 isolate as *Enterococcus faecium*. The DNA-DNA hybridization study again confirmed this identification, because the isolate showed 100% homology to an *Enterococcus faecium* reference strain, and organisms showing more than 65% homology can be considered members of the same species (Schleifer and Stackebrandt, 1983).

The antimicrobial compound produced by *E. faecium* BFE 900 was inactivated by proteases. This suggested that inhibition of indicator strains could not have resulted from organic acids, and that the inhibitory compound was proteinaceous in nature. The enzyme catalase did not affect the antimicrobial activity of supernatant, which indicated that the inhibition observed was not due to hydrogen peroxide. The antagonistic compound was not inactivated by the enzymes α-amylase and lipase, which suggested that the peptide did not contain or require a lipid or a 1,4α-bonded carbohydrate moiety for activity. Protease inactivation characteristics of the antagonistic compound compared well with those of enterocin 1146 (Parente and Hill, 1992a), enterocin 01 (Olasupo et al., 1994), and bacteriocins produced by strains *E. faecium* 7C5, RZS C5 and RZS C13 (Torri Tarelli et al., 1994; Vlaemynck et al., 1994), because these were all inactivated by α-

chymotrypsin and proteinase K. Furthermore, the antagonistic compound produced by *E. faecium* strains BFE 900, RZS C5 and RZS C13 and 7C5, as well as enterocin 1146 were all inactivated by trypsin, while enterocin 01 was not. This could indicate that the above compounds may be of similar chemical nature, but with a probable structural difference to enterocin 01.

Cell free, neutralized supernatant from *E. faecium* BFE 900 was considered to be bactericidal, because it caused a decrease in numbers of a population of sensitive *L. sake* DSM 20017 cells. It was interesting to note that although the viable count decreased overall, it did not decrease to levels less than *ca.* log 2 CFU ml^{-1}, which indicates that cells became resistant to the antimicrobial compound. Spontaneous development of resistance has been observed before but it is poorly understood (Jack et al., 1995; McMullen and Stiles, 1996). In the case of nisin resistance in *Listeria* strains, resistance was shown to result from modifications in membrane phospholipid composition (Verheul et al., 1997). The antimicrobial compounds of *E. faecium* RZS C5 and RZS C13 and enterocin P were also shown to have bactericidal activity when tested against a population of sensitive *L. monocytogenes* cells (Vlaemynck et al., 1994; Cintas et al., 1997). Similar to our study, a resistant population of *L. monocytogenes* was observed after prolonged exposure to supernatant from the *E. faecium* P13 culture which produces enterocin P (Cintas et al., 1997).

The antimicrobial compound produced by *E. faecium* BFE 900 was proteinaceous and had a bactericidal mode of action, which indicates that it is a bacteriocin. The bacteriocin was tentatively named enterocin 900.

Bacteriocin activity could be detected after heating neutralized supernatant at 100°C and 121°C. The bacteriocin was heat stable and this may be of importance when considering a potential application for this bacteriocin in heated foods. The bacteriocins from the *E. faecium* strains 7C5, RZS C5 and RZS C13 (Torri Tarelli et al., 1994; Vlaemynck et al., 1994) as well as enterocin 01 (Olasupo et al., 1994) and enterocin P (Cintas et al., 1997) were also described as heat stable, but differed from enterocin 1146

produced by *E. faecalis* DPC 1146 (Parente and Hill, 1992) which was partially or totally inactivated at temperatures above 60°C.

Enterocin 900 activity decreased at low pH. However, enterocin 900 retained activity over a wide pH range. Similar to enterocin 900, the bacteriocin enterocin P remained stable at pH values ranging between 2 and 11 (Cintas et al., 1997). Stability of a bacteriocin over a wide pH range may be of advantage in food preservation where the product is acidified, or where the pH of the food is lowered by growth of naturally occurring LAB (e.g. fermentations). On the other hand stability at a relatively high pH may be advantageous. For example, meat at typical pH 5.5 to 6.0 (Dainty and Mackey, 1992) is not suited to nisin solubility or stability (Stiles, 1996), whereas activity of enterocin 900 in this pH range is high. The broad range pH stability of enterocin 900 differs from that of other *E. faecium* bacteriocins. The bacteriocin produced by *E. faecium* 7C5 was reported to have low stability at a pH of 5.0 (Torri Tarelli et al., 1994), while stability of enterocin 1146 was low at pH >7.0 (Parente and Hill, 1992a). In addition to differences in heat stability and activity spectrum, therefore, variations in pH sensitivity may also indicate an underlying difference in the chemical structure of enterocin 900 and the other enterocins.

Enterocin 900 had a broad activity spectrum. The compound was active not only against related enterococci, but also against the potential foodborne pathogens *L. monocytogenes, C. perfringens* and *S. aureus.* The bacteriocins of enterococci are generally active against strains of *L. monocytogenes* (Giraffa, 1995) and this anti-*Listeria* activity may be explained by the close phylogenetic relatedness of enterococci and *Listeria* (Devriese and Pot, 1995). Bacteriocin-producing enterococci have been proposed for use as anti-*Listeria* agents in the dairy industry, particularly in certain soft cheeses where the pH of the rind increases as a result of mould growth to a level that allows growth of *L. monocytogenes.* Enterococci often predominate in the microbial flora of such cheeses (see chapter 1) and bacteriocin-producing strains could produce bacteriocins at levels sufficient to prevent *Listeria* growth (Giraffa et al., 1995, 1997). Many bacteriocin-producing enterococci also exhibit activity towards *Clostridium* strains. Of interest to food preservation is activity towards *C. tyrobutyricum*, a spoilage organism of

packaged cheeses that causes blowing of packages as a result of gas production. The use of bacteriocinogenic enterococci with activity against this microorganism could prevent this defect.

Enterocin 900 production occurred in the late exponential phase of growth, as is typical for bacteriocin production by most LAB (De Vuyst and Vandamme, 1994). The enterocins from *E. faecium* strains RZS C5, RZS C13 and 7C5 (Torri Tarelli et al., 1994, Vlaemynck et al., 1994), and enterocin P (Cintas et al., 1997) were all produced in the late logarithmic phase of growth. Thus maximum production of bacteriocin occurred at high cell densities, as was reported also for bacteriocins produced by other LAB (Keppler et al., 1994; van Laack et al., 1992, Vlaemynck et al., 1994; Cintas et al., 1997). Decreasing bacteriocin activity after 54 h of incubation may be explained by bacteriocin degradation due to culture proteases, or low culture pH (Parente and Hill, 1992a; Torri Tarelli et al., 1994).

Low initial medium pH (pH 3.0 to 5.0) inhibited production of enterocin 900. Maximum bacteriocin was produced in the neutral to alkaline range (pH 6.0 to 9.0). Hence, for optimizing production of enterocin 900, *E. faecium* BFE 900 should be grown at pH values between 6.0 and 9.0. The application of *E. faecium* BFE 900 as a protective culture *in situ* can be envisaged in food systems with pH > 6.0. The presence and concentrations of peptone, yeast extract and Tween 80, but not that of meat extract, influenced enterocin 900 production by *E. faecium* BFE 900. The effect of omitting Tween 80 on bacteriocin production was not as noticeable as the effect of omitting peptone or yeast extract. For enterocin 900 purification, therefore, media with either peptone or yeast extract omitted would be unsuitable for use, because bacteriocin recovery would be low. Decreasing the peptone concentration to one quarter while increasing yeast extract concentration by one half may be advantageous for enterocin 900 purification trials because this results in an acceptable bacteriocin yield, yet removes unwanted proteins. It is well known that most of the organic nitrogen in yeast extract is in peptides or amino acids with a molecular weight below 1000 Da, which can easily be eliminated during purification, while the reverse is true for peptone (Parente and Hill,

1992b). The CAA medium of Hastings et al. (1991) would be optimal for use in bacteriocin purification, because it contains yeast extract and casamino acids instead of peptone as organic nitrogen source. However, enterocin 900 production in this medium was too low (400 AU ml^{-1}) to warrant its use in purification trials.

The effect of media components on bacteriocin production may vary for production of different bacteriocins. Thus, while enterocin 900 activity was affected by concentrations of peptone, yeast extract and Tween 80 in the medium, only the peptone level influenced the production of piscicolin 61 produced by *Carnobacterium piscicola* LV61 (Schillinger et al., 1993). Production of enterocin 1146 was also dependent on levels of yeast extract and Tween 80 in the medium, but it was not affected by tryptone concentration (Parente and Hill, 1992b). For bacteriocin purification, therefore, the medium composition needs to be specifically designed to allow for maximum bacteriocin recovery with a minimum of peptones (Parente and Hill, 1992b). Our results indicated that for purification of enterocin 900, medium M7 should be best for use. Although this medium yields less bacteriocin than medium M1 (Merck commercial MRS formulation), decreased levels of peptone assure improved bacteriocin recovery at a higher degree of purity than in media with high peptone content. This medium should be evaluated for suitable bacteriocin recovery in bacteriocin purification trials.

Using small scale plasmid isolation techniques the presence of plasmid DNA could not be demonstrated in *E. faecium* BFE 900. Therefore, bacteriocin production by this isolate appeared to be encoded on the chromosome. The possibility remained that the genes responsible for bacteriocin production are located on an undetected plasmid that may exist in the cell in low copy number. However, this was considered unlikely, because the isolation method of O'Sullivan and Klaenhammer (1993) used in this study is well suited for isolation of low copy number plasmids. This result compares with that of Parente and Hill (1992a) who showed that although *E. faecium* DPC1146 harbored plasmids, bacteriocin production by the bacterium was chromosomally encoded. In addition, enterocins A, B and P (Aymerich et al., 1996; Casaus et al., 1997; Cintas et al., 1997) are chromosomally encoded. In contrast, the bacteriocin produced by *E. faecium* NA01 appeared to be plasmid-encoded (Olasupo et al., 1994). Similarly, for *E. faecalis*

226 bacteriocin production was encoded by a 5.2 kb conjugative plasmid (Villani et al., 1993). Bacteriocin production of some enterococci may, therefore, also be plasmid encoded, as is the case with bacteriocins produced by many other LAB (Klaenhammer, 1993; Jack et al., 1995).

Enterocin 900 produced by *E. faecium* BFE 900 exhibits class IIa bacteriocin characteristics. It is a proteinaceous compound that does not appear to require lipid or carbohydrate moieties for activity, it is heat stable and it has anti-*Listeria* activity. However, to classify this bacteriocin into one of the described bacteriocin classes (Klaenhammer, 1993; Nes et al., 1996), the bacteriocin would need to be purified and the primary structure determined, as has been done for enterocins A, B, P, L50A and L50B (Aymerich et al., 1996; Casaus et al., 1997; Cintas et al., 1997, 1998). Bacteriocins of enterococci such as enterocins 900, 01 and 1146, and those produced by *E. faecium* strains 7C5, RZS C5 and RZS C13 are similar in many respects (e.g., heat resistance, enzyme inactivation patterns and pH stability), yet they often differ in one of these characteristics. However, as their primary structures have not been determined, it is not known whether they differ from the enterocins that have already been purified and described. Therefore, it is essential that the amino acid sequences of these compounds be determined and compared to clarify their degree of relatedness. For this reason, purification of enterocin 900 was undertaken. Results from purification experiments are presented in Chapter 3. Cloning of the genetic determinant for enterocin 900 by reverse genetics and description of the locus responsible for the bacteriocin production are also described in Chapter 3.

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CHAPTER 3*

ATYPICAL GENETIC LOCUS FOR ENTEROCIN B PRODUCTION IN *Enterococcus faecium* BFE 900.

3.1. Introduction

Members of the genus *Enterococcus* are among the lactic acid bacteria (LAB) that are of importance in foods (Giraffa et al., 1997; Stiles and Holzapfel, 1997). Phylogenetically, they are more closely related to the genus *Carnobacterium*, than to the streptococci or lactococci from which they were derived (Devriese et al., 1993). Enterococci are used as starter cultures in some cheeses and as animal and human probiotic cultures; however, when they are present in foods adventitiously, they may be used as indicators of unsanitary handling (Jay, 1996) and they may cause spoilage of heat treated foods (Houben, 1982; André Gordon and Ahmad, 1991). Many LAB, including enterococci, produce bacteriocins. The best characterized *Enterococcus* bacteriocins are enterocins A, B and P, which are produced by *E. faecium* isolated from fermented meats (Aymerich et al., 1996; Casaus et al., 1997; Cintas et al., 1997).

Most of the well-characterized bacteriocins that have been isolated from LAB, with the notable exception of the class I lantibiotic, nisin, are class II bacteriocins (Klaenhammer, 1993; Nes et al., 1996). The class II bacteriocins are ribosomally-synthesized, small (4 to 6 kDa), heat stable peptides that, unlike the lantibiotics, do not undergo extensive posttranslational modification. Considerable emphasis has been placed on the 'pediocin-like' structure and the '*Listeria*-active' nature of the class IIa bacteriocins that contain a YGNGVXC- amino acid motif near the N-terminus of the active peptide. Enterocin B does not contain this amino acid motif; nonetheless it is

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considered to be a class IIa bacteriocin (Casaus et al., 1997). The class II bacteriocins have some distinct similarities in their genetic organization, consisting of the structural gene, followed immediately by a gene encoding the immunity protein, and genes for dedicated transporter and accessory proteins (Nes et al., 1996). Some class IIa bacteriocins are regulated and they may have an induction factor as well as regulatory genes, as demonstrated for carnobacteriocin B2 (Quadri et al., 1997) and sakacin P (Brurberg et al., 1997). An induction factor involved with regulation of enterocin A and B production has recently been described (Nilsen et al., 1998).

Some of the well-characterized bacteriocins are widespread among different bacteria. Nisins A and Z are produced by several strains of *Lactococcus lactis* subsp. *lactis* (Klaenhammer, 1993; Hansen, 1994). Pediocin PA-1/AcH was isolated separately from supposedly different strains of *Pediococcus acidilactici* (Henderson et al., 1992, Motlagh et al., 1992), and subsequently from other strains of *P. acidilactici* as well as *L. plantarum*, suggesting a widespread distribution of the genetic determinants for this bacteriocin (Ennahar et al., 1996; Rodríguez et al., 1997). Leucocin A-UAL 187, B-TA 11a and C-LA7a produced by *Leuconostoc gelidum* (Hastings et al., 1991), *Leuconostoc carnosum* (Hastings et al., 1994), and *Weissella paramesenteroides* (Hastings et al., 1996), respectively, are identical bacteriocins and were isolated from meats in Canada and South Africa; mesentericin Y105, produced by *Leuconostoc mesenteroides* isolated from a dairy product in France, differs from leucocin A-UAL 187 by only two amino acids (Hastings et al., 1991, Héchard et al., 1992).

Enterocin B production has been described for *E. faecium* T136 isolated from fermented sausage in Spain (Casaus et al., 1997). The bacteriocin enterocin 900 produced by *E. faecium* BFE 900 (described in chapter 2) is identical to enterocin B produced by *E. faecium* T136 (Casaus et al., 1997). For this reason, enterocin 900 was renamed enterocin B. In this study we characterized enterocin B production by *E. faecium* BFE 900 isolated from black olives in Germany (Franz et al., 1996) and analyzed the genetic composition of both a 2.2-kb and 12.0-kb fragment of chromosomal DNA containing the structural gene for this bacteriocin in strain BFE 900. We also report the separate cloning of the structural and immunity genes for enterocin B and the heterologous expression by a dedicated and a general secretory pathway in LAB.

3.2. Methods and Materials.

3.2.1. *Bacterial strains and culture conditions.*

The bacterial strains and plasmids used in this study are listed in Table 3.1. The bacteriocin producer *E. faecium* BFE 900 isolated from black olives (Chapter 2, Franz et al., 1996) and all other LAB were grown at 30°C in Lactobacilli MRS broth (Difco Laboratories Inc., Detroit, Michigan), except *Carnobacterium* strains which were grown in APT broth (Difco) at 25°C. For bacteriocin purification *E. faecium* BFE 900 was grown in APT broth at 30°C. *E. coli* strains were grown on a rotary shaker at 250 rpm in Luria Bertani broth (LB; Becton Dickinson, Cockeysville, Maryland) at 37°C. All cultures were incubated aerobically. Antibiotics were added as selective agents when appropriate: erythromycin (200 µg/ml) and ampicillin (150 µg/ml) for *E. coli*, erythromycin at 5 µg/ml for carnobacteria and *Lactococcus lactis* ATCC 19435, or at 50 µg/ml for *E. faecalis* ATCC 19433. Stock cultures were made in the same medium used for culturing the bacterial strain with 15% glycerol (vol./vol.) and were stored at -80°C.

3.2.2. *Bacteriocin activity assays and induction.*

Bacteriocin activity was quantified by the critical dilution method (Chapter 2, Franz et al., 1996), using neutralized culture supernatant of the producer culture grown overnight at 30°C. Indicator bacteria were inoculated (1%) into the appropriate soft (0.75%) agar medium. Unless stated otherwise, *Lactobacillus sake* DSM 20017 was used as the indicator strain for assays of bacteriocin activity. For induction experiments, *E. faecium* BFE 900 and *E. faecium* CTC492 were each grown overnight in APT broth at 30°C. The cultures were each serially diluted to extinction in APT broth using twelve tenfold dilutions. The diluted cultures were incubated at 30°C and allowed to grow until turbid. The study was repeated with an overnight culture of *E. faecium* BFE 900 that was washed three times with sterile, 0.1 M sodium phosphate buffer (pH 7.0), resuspended in 9 ml of buffer and serially diluted to extinction in APT broth as described above.

Table 3.1 Bacterial strains and plasmids used in this study.

Bacterial Strain or Plasmid		Relevant Characteristics	Reference or Source
Strains			
Enterococcus faecalis ATCC 19433		$EntB^R$	
Lactobacillus sake DSM 20017		$EntB^S$	
Carnobacterium divergens LV13		$DvnA^+$, Dvi^+, containing pCD3.4	Worobo et al. (1995)
Lactococcus lactis ATCC 19435		$EntB^S$, $CbnA^R$	
Enterococcus faecium CTC 492		*entB*$^+$, *eniB*$^+$, *entA*$^+$, *eniA*$^+$, plasmidless	M.Hugas, Centre de Technologia de Carn, Spain
Enterococcus faecium BFE 900		*entB*$^+$, *eniB*$^+$, *entA*+, plasmidless	Franz et al. (1996)
Carnobacterium piscicola LV17C		$CbnA^S$, plasmidless	Ahn and Stiles (1990)
C. piscicola LV17A		*cbnA*$^+$*cbiA*$^+$ containing pCP49	Ahn and Stiles (1990), Worobo et al. (1994)
Escherichia coli DH5a		F$^-$ *endA1 hsdR17* (r_k-m_k+) *supE44*thi-1 λ^- *recA1 gyrA96 relA1 Δ (argF-lacZYA)* U169 Φ80*dlacZΔM15*	BRL Life Technologies Inc.
E. coli MH1		MC1061 derivative; *ara*D139 *lacX74 galU galK hsr hsm*$^+$ *strA*	Casadaban and Cohen (1980)
Plasmids:	pUC118	*lacZ'* Amp^r, 3.2 kb	Vieira and Messing (1987)
	pMG36e	Expression vector, Em^r, 3.6-kb	Van de Guchte et al. (1989)
	pRW19e	pMG36e derivative containing divergicin A structural and immunity genes	McCormick et al. (1996)
	pCMAP01	pUC118 containing 12.0-kb *Eco*RI fragment; *entB*$^+$/*eniB*$^+$	This study
	pCMAP02	pUC118 containing 2.2-kb *Hin*dIII fragment; *entB*$^+$/*eniB*$^+$	This study
	pCMAP03	pRW19e containing 200-bp *Hin*dIII-*Kpn*I PCR product insert that creates the divergicin A signal peptide::enterocin B gene fusion	This study
	pCMAP04	pMG36e containing 2.1-kb *Xba*I-*Pst*I fragment, *entB*$^+$/*eniB*$^+$	This study
	pCMAP05	pMG36e containing 197-bp *Xba*I-*Hin*dIII PCR product of *eniB*	This study
	pCMAP06	pUC118 containing 138-bp *Xba*I-*Hin*dIII PCR product of a part of the gene coding for mature enterocin A.	This study

Neutralized culture supernatant was prepared from all dilutions that grew, and bacteriocin activity was determined by the critical dilution assay.

3.2.3. *Enterocin B purification and Tricine-SDS-PAGE electrophoresis.*

For enterocin B purification *E. faecium* BFE 900 was grown aerobically with gentle stirring in 3 liters of APT broth without pH control at 30°C for 18 h. After growth, the culture was heated at 70°C for 30 min to inactivate proteases, and centrifuged at 11,700 x *g* for 40 min. The supernatant fraction (fraction I, 3 liters) was loaded onto an Amberlite XAD-8 column (150 x 75 mm; BDH Chemicals Ltd., Poole, UK) and washed with 2.5 liters of 0.1% TFA, followed by 2 liters of 30% ethanol in 0.1% TFA. Bacteriocin was eluted with 1.5 liters of 60% ethanol in 0.1% TFA. The eluate was adjusted to pH 5.0 with 30% ammonium hydroxide solution and concentrated to 100 ml by rotary evaporation (fraction II). This was loaded onto a SP Sepharose® Fast Flow cation exchange column (Pharmacia Biotech, Baie D'Urfé, PQ, Canada; 110 x 13 mm) pre-equilibrated with 20 mM sodium acetate buffer at pH 5.0 (SAB). The column was washed with 100 ml SAB, followed by 60 ml of SAB with 100 mM sodium chloride added. The bacteriocin eluted with 60 ml of SAB containing 500 mM sodium chloride. The bacteriocin containing eluate was desalted using a Sep Pak C18 reverse-phase column (Waters Limited, Mississauga, ON, Canada) which was conditioned according to the manufacturer's instructions. The column was washed with 20 ml of distilled water and 10 ml of 40% ethanol. Bacteriocin was eluted with 10 ml of 70% ethanol and subsequently freeze dried. The freeze dried protein was resuspended in 1.5 ml of 0.1% TFA (fraction III) and purified by high pressure liquid chromatography (HPLC) by injecting 200 µl aliquots on a C_{18} reverse phase column [Waters Delta-Pak, 8 x 100 mm; 15 µm particle size; 300Å pore size; flow rate 1.0 ml/min; mobile phase, 0.1 % TFA (A) and 95% ethanol (B)]. Bacteriocin was eluted by a gradient method, first 35 to 60% solvent B in 7 min and then 60 to 70% solvent B in 10 min. Fractions were monitored for absorbance at A_{218}. HPLC-purified enterocin was freeze-dried and resuspended in 200 µl 0.1% TFA. Bacteriocin activity of all fractions was determined by the critical dilution

method against *L. sake* DSM 20017. Protein concentration of the fractions was determined by a modified Lowry method (Hastings et al., 1991).

To estimate the size and to confirm the purity of enterocin B, duplicate samples of HPLC-purified enterocin B were subjected to electrophoresis together with low molecular weight markers (Promega, Madison, Wisconsin) on Tricine-SDS-PAGE gels according to the methods of Schägger and von Jagow (1987). The gel was cut into two halves, each half containing a purified enterocin sample. One half was fixed, washed with distilled water to remove the SDS, and overlayered with soft (0.75%) MRS agar inoculated (1%) with sensitive *L. sake* DSM 20017 indicator cells by methods described earlier (Worobo et al., 1995). The plate was incubated at 30°C overnight. The other half was stained with Coomassie brilliant blue (Bio-Rad laboratories Ltd., Mississauga. Ontario).

3.2.4. *N-terminal amino acid sequencing and mass spectrometry.*

Purified enterocin B was subjected to Edman degradation analysis by methods described earlier (Worobo et al., 1994). The mass spectra of purified enterocin B were obtained by direct injection of a solution (50% aqueous acetonitrile, 0.1% TFA) onto a VG ZabSpec sector instrument (Fisons, Manchester, UK) with an electrospray ionization source.

The mass of both enterocin B and enterocin A (see below) was determined from the supernatant of cultures of *E. faecium* strains BFE 900 and CTC 492 also by matrix-assisted laser desorption/ionization - time of flight (MALDI-TOF) mass spectrometry by methods of Rose et al. (1998). For this, supernatant was first boiled for 5 min to inactivate cells, and 0.5 µl of supernatant was spotted onto a MALDI-TOF probe. The sample was air-dried, washed for 30 s by immersion into sterile distilled water and again allowed to air dry. A 0.5 µl volume of matrix (two parts 0.1% TFA and one part acetonitrile, saturated with sinapinic acid) was spotted onto the sample and air dried. The sample was analyzed on a linear Bruker Proflex™ III MALDI-TOF equipped with delayed extraction technology and a 125 cm flight tube (Bruker Analytical Systems, Billerica, Massachusetts). All spectra were acquired in positive ion linear mode with a

nitrogen laser (λ = 337 nm) for desorption/ionization of the samples and an acceleration voltage of 20 kV. The spectra are representative of 60 consecutive laser shots and were smoothed. External mass calibration was performed with two points that bracket the mass range of the analyte. Angiotensin (MH^+ = 1046.542) and bovine insulin (MH^+ = 5734.557) were used as calibrants (Rose et al., 1998).

3.2.5. *DNA isolation, manipulation and hybridization.*

Large scale plasmid DNA preparations (Worobo et al., 1994), small scale plasmid isolations from *E. coli* (Sambrook et al., 1989) and LAB (van Belkum and Stiles, 1995), as well as chromosomal DNA isolation (Quadri et al., 1994) were done by established techniques. DNA fragments were recovered from agarose gels using either Geneclean II (Bio 101 Inc., La Jolla, CA), or QIAEX II (QIAGEN, Chatsworth, California). Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase (New England Biolabs, Missisauga, Ontario, Canada) and Klenow enzyme (Promega, Madison, Wisconsin) were used as recommended by the suppliers. DNA manipulations, cloning and hybridizations were done as described by Sambrook et al. (1989). Competent cells of *E. coli* were prepared and transformed according to the one-step method of Chung et al. (1989). Recombinant pMG36e plasmids were first transformed into *E. coli* MH1 before being transformed into LAB. Carnobacteria and *L. lactis* ATCC 19435 cells were transformed by electroporation (Worobo et al., 1995), while *E. faecalis* ATCC 19433 cells and *L. sake* DSM 20017 cells were electroporated according to the methods of Cruz-Rodz and Gilmore (1990) and Berthier et al. (1996).

Southern and colony blot hybridizations with Hybond N (Amersham Canada, Oakville, Ontario, Canada) nylon membranes were done by standard methods (Sambrook et al., 1989). A 32-mer degenerate probe CFR-01 (5'-GAA AAT GAT CAT (CA)G(TA) ATG CC(TA) AAT GAA CT(TA) AA-3'), corresponding to the N-terminus of the enterocin B structural gene (*entB*), was used to identify *entB* in Southern and colony blot hybridizations. Oligonucleotides were synthesized on an Applied Biosystems 391 PCR MATE synthesizer (Applied Biosystems, Foster City, California) and used without further purification. Oligonucleotides were end-labeled with [γ-^{32}P]-ATP (Amersham

Canada, Oakville, Ontario). Hybridizations were done in 2 x SSPE hybridization solution (Sambrook et al., 1989) at 44°C with 5% formamide using probe CFR-01.

3.2.6. *DNA and amino acid sequence analysis.*

DNA was sequenced bidirectionally and analyzed in an Applied Biosystems 373A Sequencer using fluorescent dyedeoxy-chain terminators. The recombinant plasmids pCMAP01 and pCMAP02 (Table 3.1) were used as a templates. Primers used for sequencing were forward and reverse universal primers for the pUC plasmid series. In addition, specific oligonucleotides were synthesized on an Applied Biosystems 391 PCR MATE synthesizer, and used for sequencing in a primer-walking strategy. The nucleotide sequence was analyzed using the DNAStrider program (version 1.2).

3.2.7. *Nucleotide sequence accession number.*

The nucleotide sequences of both the 2.2-kb and 12.0-kb cloned chromosomal DNA fragments from *E. faecium* BFE 900 were submitted to GenBank (Los Alamos, New Mexico) and were given the accession number AF076604 for the 2.2.-kb *Hin*dIII fragment and xxx for the 12.0-kb *Eco*RI fragment, respectively.

3.2.8. *PCR amplification.*

Reaction1: Primer CFR-02 (5'–TAT ATC TAG AAA ATA TTA TGG AAA TGG AGT GTA T-3') was complementary to the YGNGVXC-consensus motif at the 5' end of the gene encoding mature enterocin A, while CFR-03 (5'-TAT ACT GCA GGC ACT TCC CTG GAA TTG CTC C-3') was complementary to the 3' end of the enterocin A gene (Aymerich et al., 1996). Primers CFR-02 and CFR-03 contained *Xba*I and *Pst*I restriction sites, respectively (underlined in each sequence).

Reaction 2: The open reading frame (ORF) *eniB* located adjacent to the enterocin structural gene was amplified by PCR using the primers CFR-04 (5'-TTA AGC TTT TAC GAG TTT TTT CTC TTC T-3') and CFR-05 (5'-AAT CTA GAA AAG AGA

GGA TGT TTA TAT T-3'), complementary to the 5' and 3' ends of *eniB*, respectively. Primer CFR-04 contains a *Hin*dIII restriction site, while CFR-05 contains a *Xba*I restriction site (underlined in each sequence above).

Reaction 3: The primers CFR-06 (5'CCC AAG CTT CTG CTG AAA ATG ATC ACA GAA TGC CTA A-3') and CFR-07 (5'CCC CTG CAG CAT GCT TAG TTG CAT TTA GAG TAT AC-3') were used to create a divergicin A signal peptide::enterocin B fusion construct, similar to McCormick et al. (1996) for a divergicin A signal peptide::carnobacteriocin B2 fusion construct. Primer CFR-07 contained adjacent *Pst*I and *Sph*I restriction sites (underlined) in which one base is common to both restriction enzyme sites, and it is complementary to the 3' end of the enterocin B structural gene on pCMAP02. CFR-06 contained a *Hin*dIII restriction site (underlined) which together with the next 5 nucleotides (CTGCT) encodes the carboxy terminus of the divergicin A signal peptide (Worobo et al., 1995; McCormick et al., 1996). The nucleotides which follow CTGCT encode the amino terminus of mature enterocin B. Primer CFR-06 created an in-frame fusion of the 3' end of the divergicin A signal peptide and the enterocin B structural gene.

For all PCR reactions DNA was amplified in 100 µl using a temperature cycler (Omnigene, InterSciences Inc., Markham, Ontario, Canada). PCR mixtures contained 1.0 µM of each of the respective primers, 200 µM of dNTPs, 3 mM $MgCl_2$, 2.5 units of *Taq* DNA polymerase (Perkin Elmer) and 1 x reaction buffer (Perkin Elmer). *E. faecium* BFE 900 chromosomal DNA was used as template DNA for reaction 1 and pCMAP02 for reactions 2 and 3. DNA was amplified in 32 cycles (denaturation, 94°C, 1 min; annealing, 56°C for reaction 1, 48°C for reaction 2 and 54°C for reaction 3, 1 min; extension, 72°C, 1 min). The PCR products were cloned into pUC118 for sequencing and to confirm the fidelity of the reactions.

3.2.9 *Expression of enterocin B structural and immunity genes in heterologous hosts.*

Plasmid pRW19e is a derivative of pMG36e and contains the divergicin A structural gene bearing a signal peptide as well as the divergicin immunity gene. Digestion with *Hin*dIII and *Kpn*I removes the 3' end of the divergicin A signal peptide

and the divergicin A structural and immunity genes (McCormick et al., 1996). The PCR product of reaction 3 above was cloned into pUC118. A divergicin A signal peptide::enterocin B structural gene fusion was created by excising the PCR product from pUC118 with *Hin*dIII and *Kpn*I, and inserting it into these restriction sites of pRW19e, resulting in plasmid pCMAP03 (Table 3.1).

E. faecalis ATCC 19433 was transformed with pCMAP03 and tested for bacteriocin production, using *L. sake* DSM 20017 containing pMG36e as the bacteriocin-sensitive indicator. *E. faecalis* ATCC 19433 and *E. faecium* BFE 900 both containing pMG36e were used as negative and positive controls, respectively. *C. piscicola* LV17A containing pCP49 ($CbnA^+/Imm^+$) was transformed with pCMAP04 and tested for bacteriocin production by the deferred antagonism assay (Ahn and Stiles, 1990). *C. piscicola* LV17A containing pMG36e and *C. piscicola* LV17C ($CbnA^S/EntB^S$) containing pCMAP04 were used as negative controls. *L. lactis* ATCC 19435 containing pMG36e was used as an enterocin B-sensitive, carnobacteriocin A-resistant indicator.

Immunity of *L. sake* DSM 20017 containing either pCMAP04 or pCMAP05 (Table 3.1) was determined with the transformants as indicators in deferred inhibition assays with *E. faecalis* ATCC 19433 containing pCMAP03 as the producer strain. *L. sake* DSM 20017 containing pMG36e was used as enterocin B-sensitive control. Presence of recombinant pCMAP plasmids in transformed strains was confirmed by small scale plasmid isolation and electrophoresis on 0.7% agarose gels in TAE buffer (Sambrook et al., 1989).

3.2.10. *Tests for multiple bacteriocin production.*

To investigate the possibility that *E. faecium* BFE 900 produced more than one bacteriocin, *E. faecium* BFE 900 containing pMG36e and *E. faecalis* ATCC 19433 containing pCMAP03 ($EntB^+$) were used in the deferred inhibition assay against the indicator strains: *L. sake* DSM 20017, *C. piscicola* LV17C and *C. divergens* LV13, all of which contained pMG36e (Table 3.1). For deferred inhibition assays the *E. faecium* and *E. faecalis* strains were spotted onto the same APT agar plate and were grown overnight

at 30°C, before overlayering with the indicator, by methods described earlier (section 3.2.2.).

3.3. Results.

3.3.1. *Induction.*

Bacteriocin activity (3,200 AU ml^{-1}) against *L. sake* DSM 20017 was detected in cell free, neutralized supernatant of all cultures of *E. faecium* strain BFE 900 that grew after dilution to extinction, including cells that were washed with sodium phosphate buffer before dilution in APT broth. In contrast, for strain CTC 492, bacteriocin activity (3,200 AU ml^{-1}) was observed only in cultures that were diluted to 10^{-4} and then re-grown. For strain CTC 492, cultures that were diluted higher than 10^{-4} and that grew after dilution (dilutions 10^{-5} to 10^{-9}) did not show bacteriocin activity in the supernatant.

3.3.2. *Enterocin B purification and mass spectral analysis.*

Enterocin B was purified from 3 liters of culture supernatant with a 1,205-fold purification (Table 3.2). Approximately 1 mg of pure enterocin B was recovered. Overall recovery was 1.1% of the activity detected in the culture supernatant. Enterocin B was eluted as a single peak on a C_{18} reverse phase column. Purity of the HPLC fraction was confirmed by Tricine-SDS-PAGE gel electrophoresis, and a single band located between the 2.5 and 6.2 kDa molecular weight markers was obtained (Fig. 3.1), which showed antimicrobial activity (result not shown) when the gel was overlayered with *L. sake* DSM 20017. N-terminal amino acid analysis of the HPLC purified enterocin B revealed the following 53 amino acid sequence: Glu-Asn-Asp-His-Arg-Met-Pro-Asn-Glu-Leu-Asn-Arg-Pro-Asn-Asn-Leu-Ser-Lys-Gly-Gly-Ala-Lys-Xaa-Gly-Ala-Ala-Ile-Ala-Gly-Gly-Leu-Phe-Gly-Ile-Pro-Lys-Gly-Pro-Leu-Ala-Trp-Ala-Ala-Gly-Leu-Ala-Asn-Val-Tyr-Ser-Leu (Lys)-Xaa-Leu (Asn).

Table 3.2 Purification of enterocin B produced by *E. faecium* BFE 900 in 3 liters APT broth culture at 30°C.

Method	Fraction	Volume (ml)	Total activity (AU)	Activity Recovery (%)	Total Protein (mg)	Specific Activity (AU/mg)	Purification (fold)
Culturing and harvesting of supernatant	Fraction I	3000	1.92×10^7	100	1.32×10^5	1.46×10^2	1
Hydrophobic interaction chromatography, rotary evaporation	Fraction II	100	1.02×10^7	53	3.61×10^2	2.82×10^4	193
cation exchange, Sep Pak desalting and freeze drying	Fraction III	1.5	3.07×10^5	1.6	2.33	1.32×10^5	904
Reverse phase HPLC purified Enterocin B	Fraction IV	1	2.04×10^5	1	1.16	1.76×10^5	1205

Interpretation of the nucleotide sequence indicated that the amino acids at positions 23 and 52 were cysteine. The nucleotide sequence also showed that amino acids 51 and 53 were incorrectly identified as leucine, and that they are lysine and asparagine, as indicated above in parentheses.

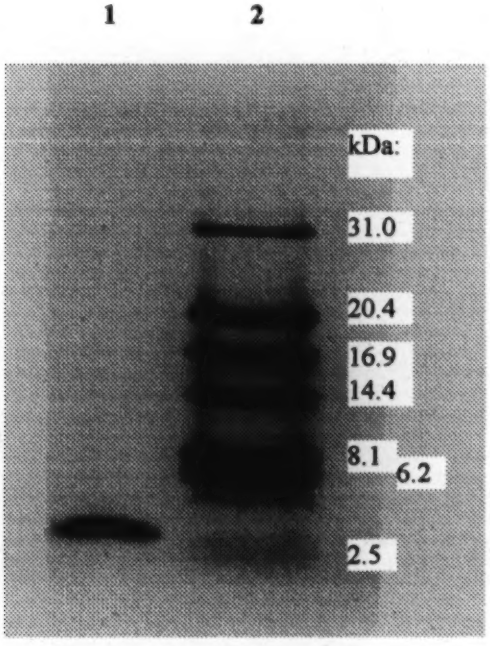

Figure 3.1 Tricine-SDS-PAGE gel showing HPLC purified enterocin B (lane 1) and molecular weight markers (lane 2).

The average molecular mass of enterocin B determined by mass spectral analysis was 5463.0 +/- 1 Da. (Fig. 3.2). Using MALDI-TOF mass spectral analysis a compound with a mass ranging from 5476 to 5480 Da. could be detected in the supernatant of the *E. faecium* BFE 900 culture, which approximates the mass of enterocin B (see above) assuming that oxidation of a methionine residue occurred. In addition, a compound with a mass ranging from 4840 to 4841 Da. was detected in the supernatant, which approximates the mass reported for enterocin A (Aymerich et al., 1996), also assuming that oxidation of a methionine residue occurred (results not shown). Two compounds with similar molecular mass could be identified in mass spectra of *E. faecium* CTC 492 culture supernatant (results not shown).

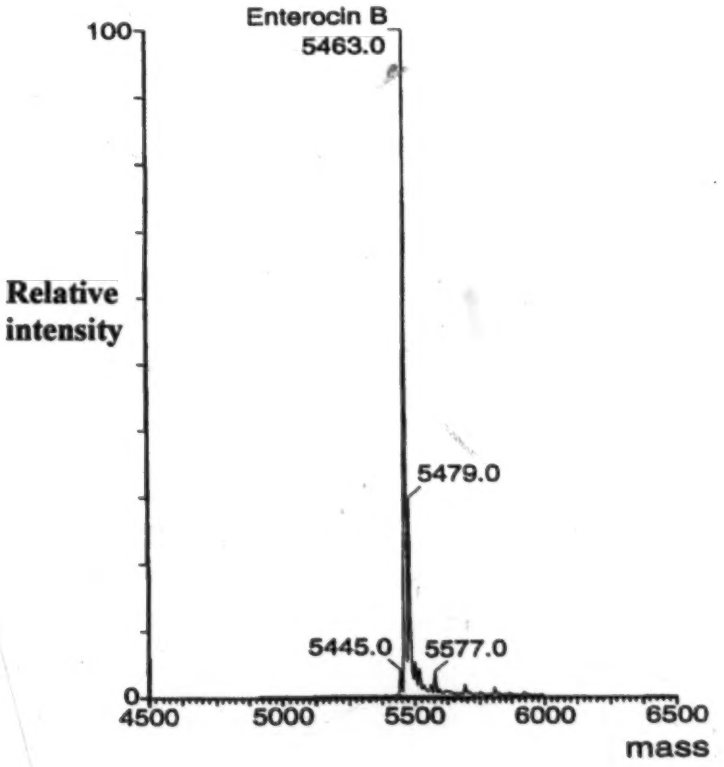

Figure 3.2 Electrospray mass spectrum of enterocin B.

Using MALDI-TOF mass spectrometry, the enterocin A and B compounds could be detected in supernatants of all *E. faecium* strain BFE 900 cultures that grew after dilution to extinction in induction studies. In contrast, enterocin B could not be detected in the supernatant of strain CTC 492 cultures that grew after dilution to 10^{-5} or higher (results not shown).

3.3.3. *Nucleotide sequence and identification of the enterocin B structural gene.*

Plasmid DNA was not isolated from *E. faecium* BFE 900 with small (Chapter 2; Franz et al., 1996) and large scale (this study) plasmid isolation methods. Probe CFR-01 hybridized to both a 2.2-kb *Hin*dIII and a 12.0-kb *Eco*RI chromosomal DNA fragment. These fragments were cloned separately into pUC118 and completely sequenced in both directions. The nucleotide sequence of the 2.2-kb fragment is shown in Fig. 3.3 and the ORFs located on the 12.0-kb fragment are shown in Fig. 3.4. Analysis of the nucleotide sequence of the 2.2-kb fragment revealed eight possible ORFs, two oriented in the 5' to 3' direction (*orf1* and *entB*) and six in the opposite direction (*orf2 to orf6* and *eniB*, Figs. 3.3 and 3.4). The translation of the *entB* ORF matched the amino acid sequence determined by Edman degradation analysis for enterocin B. The first amino acid of the N-terminal sequence (Glu) matches the nineteenth amino acid of the nucleotide sequence. The enterocin prepeptide consists of an 18 amino acid N-terminal extension ending in a double-glycine cleavage site, and a 53 amino acid bacteriocin identical to the prepeptide reported for enterocin B from *E. faecium* T136 (Casaus et al., 1997). A probable ribosome binding site (RBS) for the enterocin B structural gene (AAAGGAG) was located eleven bases upstream of the initiation codon. Two possible –10 and –35 promoter sequences, i.e. -10 (1 or 2) and –35 (1 or 2), were detected upstream of the structural gene (Fig. 3.3).

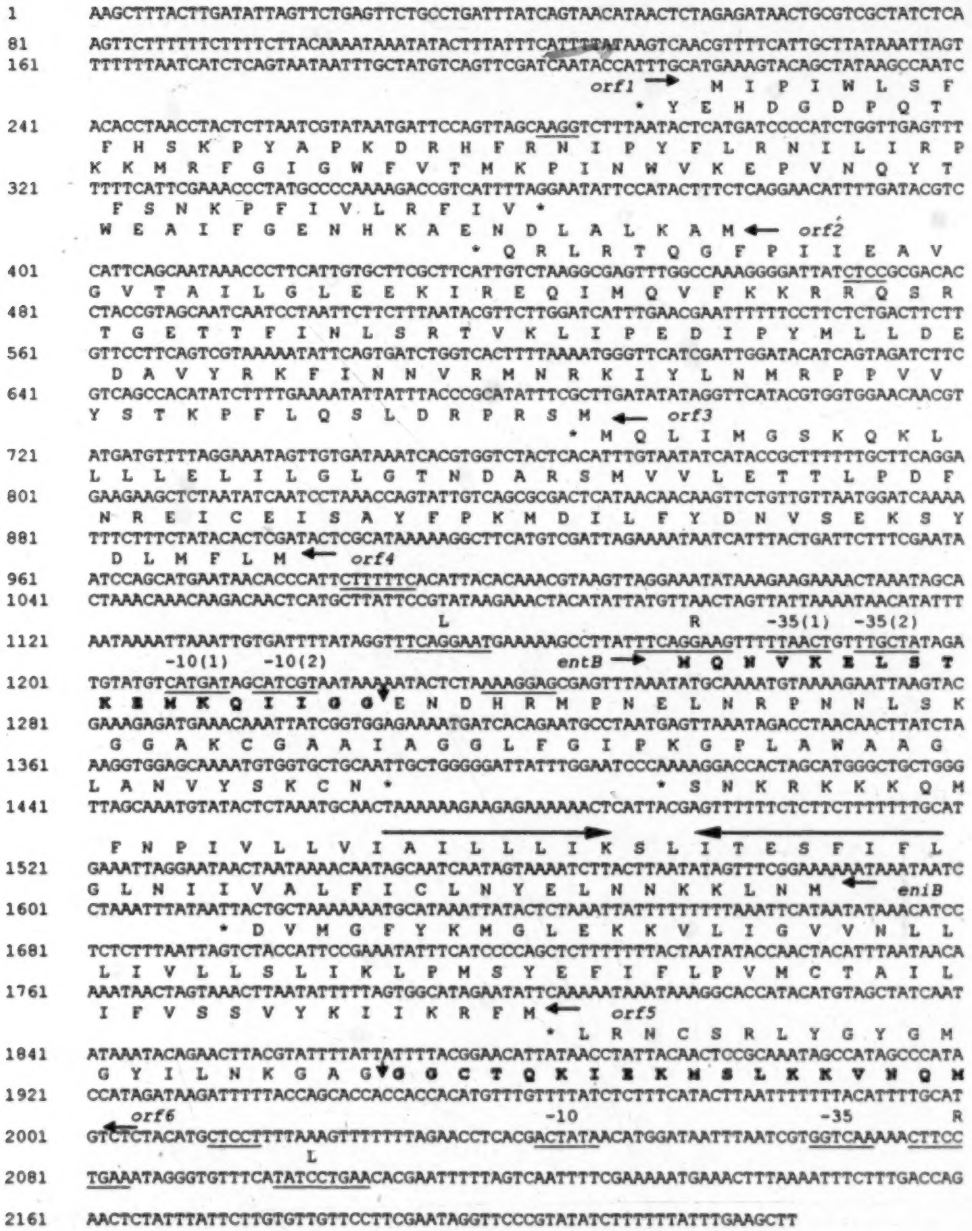

Figure 3.3 Single stranded nucleotide sequence of a 2.2-kb *Hin*dIII fragment of *E. faecium* BFE 900 chromosomal DNA. The sequence shows eight open reading frames (*orf1-6, eniB* and *entB*) with their putative ribosome binding sites underlined. The translation products of ORFs are shown above the nucleotide sequence. Double-glycine-type leader peptides are shown in bold print and cleavage sites are indicated with a vertical arrow. Reversed horizontal arrows indicate a *rho*-independent terminator site Right (R) and left (L) direct repeats of conserved regulatory-like box sequences are underlined.

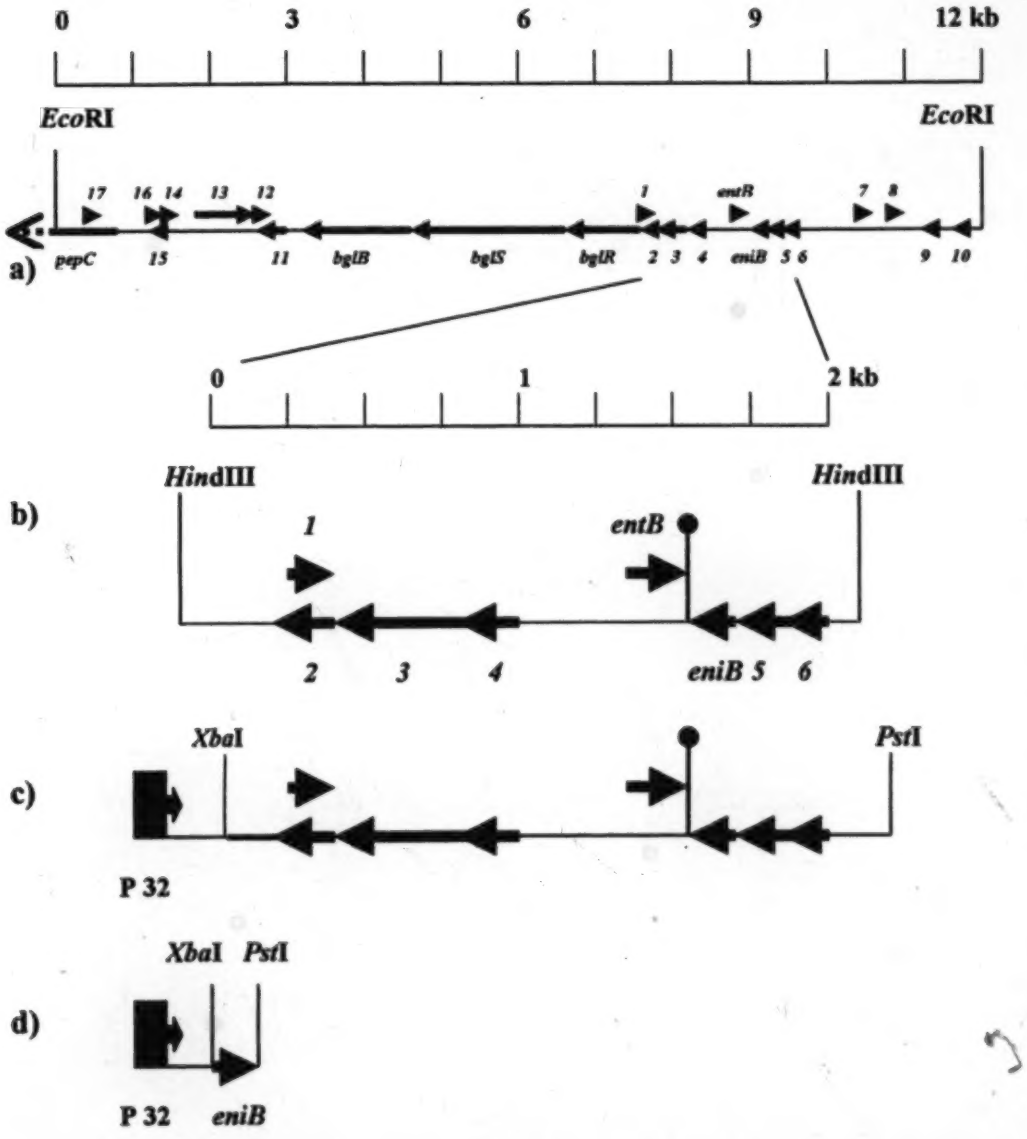

Figure 3.4 Organization of genes on the cloned 12.0-kb and 2.2-kb chromosomal fragments from *Enterococcus faecium* BFE 900 associated with enterocin B production. Constructs used for enterocin B immunity testing are shown. A terminator structure is indicated by a stem-loop vertical line and the P 32 promoter by a box and arrow. Chromosomal fragments and constructs are contained in plasmids pCMAP01 (a), pCMAP02 (b), pCMAP04 (c) and pCMAP05 (d).

A conserved regulatory-like box, consisting of the direct repeat sequences TTCAGGAAT (left) and TTCAGGAAG (right), separated by twelve nucleotides, was located upstream of the –35 site. An imperfect inverted repeat starting with the third base of the last amino acid codon (Asn) of the enterocin structural gene had the characteristics of a possible *rho*-independent terminator (Fig. 3.3). A second conserved regulatory-like box consisting of the sequences TTCAGGATA (right) and TTCAGGAAG (left), separated by twelve nucleotides, was located upstream of *orf6* (Fig. 3.3).

3.3.4. *Amino acid homology.*

EntB from *E. faecium* BFE 900 is identical to that produced by *E. faecium* T136 (Casaus et al., 1997). A search of the protein data banks revealed that none of the ORFs on the cloned 2.2-kb fragment (Fig. 3.3) or the 12.0-kb fragment showed homology to reported bacteriocin immunity proteins, but immunity proteins in general share little homology (Aymerich et al., 1996; Nes et al., 1996). The protein product of *orf6* has homology to the N-terminal extensions of the sakacin A prepeptide and the precarnobacteriocins A, B2 and BM1. The first 18 amino acids of this protein are characteristic of bacteriocin leader peptides of the double-glycine-type (Fig. 3.5); however, the mature peptide following the N-terminal extension is unlikely to encode a bacteriocin, because it contains only 21 amino acids. *Orf6* may encode an inducer peptide (see below). The putative protein products of *orf* 2, 3 and 4 all show low homology (7.2 %, 7.2 % and 6.8 % identity, respectively) to a UV resistance protein produced by *E. faecalis* that is transcribed from a single ORF in that strain (Ozawa et al., 1997). The putative protein products of *orf1* and *orf5* showed no clear homology to reported amino acid sequences.

The nucleotide sequence from the 12.0-kb *Eco*RI chromosomal fragment consists of approximately 3-kb of DNA sequenced downstream and 9-kb sequenced upstream of the enterocin B structural gene. Analysis of the DNA sequence downstream of *entB* revealed the presence of four additional ORFs (Fig. 3.4). The putative protein products of these ORFs did not have homology to reported amino acid sequences in the data banks. Analysis of the DNA sequence upstream of *entB* revealed the presence of eleven

EntB	M Q N V K E L S T K E M K Q I I G G
CbnA	M N N V K E L S I K E M Q Q V T G G
SakA	M E K F I E L S L K E V T A I T G G
CbnB2	M N S V K E L N V K E M K Q L H G G
CbnBM1	M K S V K E L N K K E M Q Q I N G G
Orf6	M Q N V K K L S M K E I K Q T C G G
Consensus	V K E L S K E M Q I G G
Consensus 1 Håvarstein et al. (1994)	L S E L I G G
Consensus 2 Håvarstein et al. (1994)	* * # # # * *

Figure 3.5 Amino acid sequences of double-glycine-type leader peptides of enterocin B (EntB), carnobacteriocin A (CbnA), carnobacteriocin B2 (Cbn2), carnobacteriocin BM1 (CbnBM1) sakacin A (sakA), and Orf6. Common amino acids are shown in bold print; consensus between these leader peptides and the consensus previously reported by Havarstein et al. (1994) are shown, * = hydrophobic residue, # = hydrophilic residue.

additional ORFs, six of which were in the opposite orientation to the *entB* structural gene. The first (*bglR*) located immediately upstream of *orf1* encoded a protein of 281 amino acids, which showed high homology to transcription antiterminator proteins of the BglG family such as LicT of *B. subtilis* (37.5 % identity), BglG of *E. coli* K12 (34.5 % identity), ArbG of *Erwinia chrysanthemi* (31.8 % identity), and BglR of *L. lactis* (28.3 %

identity) (Schnetz et al., 1987; Zukowski et al., 1990; El Hassouni et al., 1992; Bardowski et al., 1995; Schnetz et al., 1996). The ORF immediately following (*bglS*) showed homology to the β-glucoside-specific transport proteins (enzyme II^{Bgl}) such as BglS of *E. coli* K12 (31.7 % identity) and ArbF of *E. chrysanthemi* (33.0 % identity) (Schnetz et al., 1987; El Hassouni et al., 1992). The next ORF (*bglB*) has homology to phospho-β-glucosidase hydrolyzing enzymes such as BglH of *B. subtilis* (45.8% identity), ArbB of *E. chrysanthemi* (43.2 % identity), and BglB of *E. coli* K12 (41.5 % identity) (Schnetz et al., 1987; El Hassouni et al., 1992; Le Coq et al., 1995). The putative protein products of the seven ORFs (*orf11* to *orf17*) located downstream of *bglB* did not show clear homology to reported amino acid sequences. The 5' end of the ORF *pepC* was detected at the proximal end of the cloned fragment, but the 3' end of this ORF was not located within the cloned fragment. This truncated ORF showed homology to cysteine aminopeptidase proteins such as PepC of *S. thermophilus* (30.3% identity) and *L. lactis* subsp. *cremoris* (28.7% identity) (Chapot-Chartier et al., 1993, 1994). These identity values are based on the amino acid sequence derived from the truncated *E. faecium* BFE 900 PepC gene present on the cloned fragment in this study, and can be expected to be greater for the entire *E. faecium* BFE 900 PepC protein.

3.3.5. *Heterologous expression of enterocin B.*

E. faecalis ATCC 19433 was electrotransformed with plasmid pCMAP03 (Table 3.1) for heterologous bacteriocin expression by the *sec*-pathway, and *L. sake* DSM 20017 containing pMG36e was used as the indicator strain. Transformants containing pCMAP03 exhibited activity against the indicator, whereas *E. faecalis* ATCC 19433 containing pMG36e did not (Fig. 3.6a); however, the zone of antimicrobial activity produced by wild type *E. faecium* BFE 900 containing pMG36e was noticeably larger than that produced by the heterologous host containing pCMAP03 (Fig. 3.6a).

L. lactis ATCC 19435 was used as the indicator for heterologous bacteriocin expression by the dedicated secretion machinery, because this strain is sensitive to EntB but not to CbnA (Fig. 3.6b). Transformants of *C. piscicola* LV17A with plasmid

pCMAP04 exhibited antimicrobial activity against this indicator, whereas transformants of *C. piscicola* LV17C containing plasmid pCMAP04 did not (Fig. 3.6b).

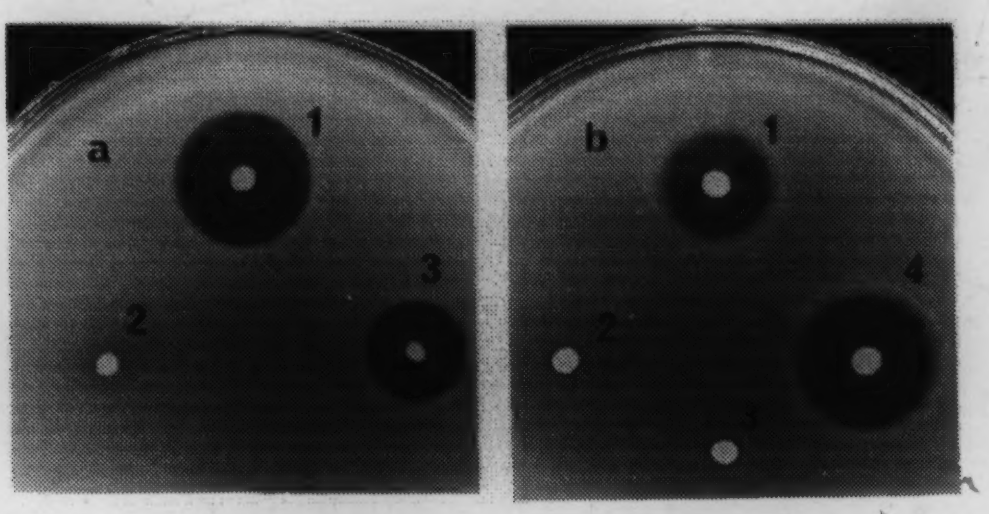

Figure 3.6 Deferred inhibition tests against *L. sake* DSM 20017 containing pMG36e (a) with *E. faecium* BFE900 (1), *E. faecalis* ATCC 19433 containing pMG36e (2) and *E. faecalis* ATCC 19433 containing pCMAP03 (3); against *L. lactis* ATCC 19435 containing pMG36e (b) with *E. faecium* BFE900 (1), *C. piscicola* LV17A (2) and *C. piscicola* LV17C (3) (all containing pMG36) and *C. piscicola* LV17A containing pCMAP04 (4).

3.3.6. *Multiple bacteriocin production.*

The nucleotide sequence from the PCR amplicon in pCMAP06 (Table 3.1) obtained from PCR reaction 1 encoded amino acids identical to the last 42 amino acids of the enterocin A gene, as determined previously by Aymerich et al. (1996). Codon usage was identical in the sequence derived from chromosomal DNA of *E. faecium* BFE 900 to that reported for *E. faecium* CTC492 by Aymerich et al. (1996) (results not shown).

Production of additional bacteriocin was also suggested by deferred inhibition tests with *E. faecium* BFE 900 and an EntB-producing *E. faecalis* clone. These tests showed that although a zone of activity was obtained with both producers when *L. sake* DSM 20017 was used as indicator, the zone of inhibition obtained with the *E. faecalis* clone was noticeably reduced (Fig. 3.7). While inhibition zones were obtained with the *E. faecium* BFE 900 strain, no such zones could be obtained with the *E. faecalis* clone when *C. piscicola* LV17C and *C. piscicola* UAL26 were used as indicator strains (Fig. 3.7).

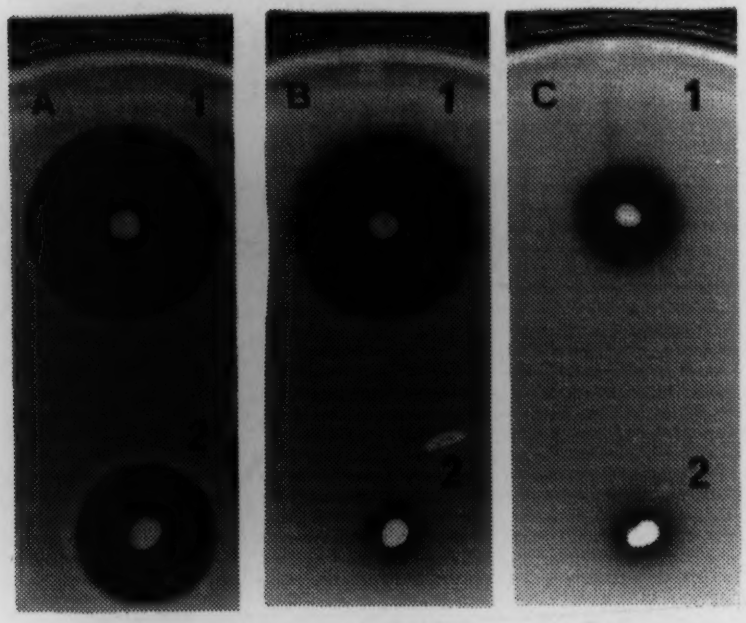

Figure 3.7 Deferred inhibition test with *E. faecium* BFE 900 containing pMG36e (1) and *E. faecalis* ATCC19433 containing pCMAP03 ($EntB^+$) (2) against *L. sake* DSM 20017 (a), *C. piscicola* UAL26 (b) and *C. divergens* LV17C (c).

3.3.7. *Immunity gene.*

The possibility that one of the putative ORFs shown in Fig. 3.4 encoded the immunity protein for EntB was investigated. When *L.* sake DSM 20017 containing pCMAP04 was used as an indicator in a deferred inhibition test with *E. faecalis* containing pCMAP03 as a producer, a smaller zone of inhibition resulted compared with that of the *L. sake* control containing pMG36e (Fig. 3.8). This indicated that one or more ORFs on the 2.2-kb fragment was involved in immunity.

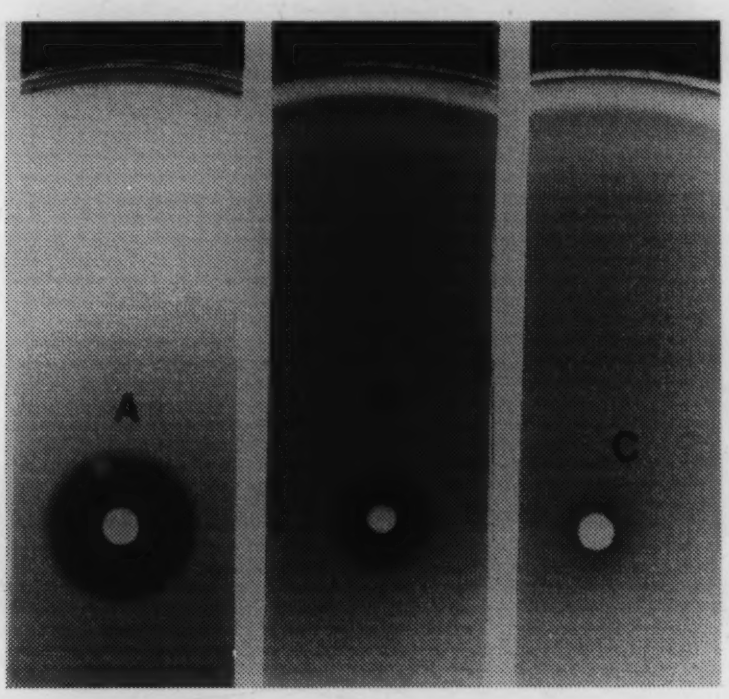

Figure 3.8 Deferred inhibition test with *E. faecalis* ATCC 19433 containing pCMAP03 ($EntB^+$) against *L. sake* DSM 20017 (A) and *L. sake* DSM 20017 containing plasmid pCMAP04 (B) and pCMAP05 (C).

The ORF *eniB* was considered a likely candidate, because it is located adjacent to the enterocin structural gene albeit in opposite orientation (Figs. 3.3 and 3.4), and it has a relatively strong RBS. This ORF was amplified by PCR and cloned into pMG36e (pCMAP05) in correct orientation to the P32 promoter for expression (Fig. 3.4), and transformed into *L. sake* DSM 20017 for use as an indicator in the deferred antagonism assay with *E. faecalis* ATCC 19433 containing pCMAP03. The absence of a zone of inhibition in this assay indicated that *eniB* is the immunity gene for EntB (Fig. 3.8). This gene encoded a putative protein product of 58 amino acids that has charged amino acids at both its ends. Using the PepTool protein structure prediction software (version 1.0.0B1, BioTools, Edmonton, Alberta) the central region (residues 9 to 49) was predicted to form an α-helix that could insert into the membrane. The polar ends were assumed to be random coil.

3.4. Discussion.

A modified MRS broth medium (medium M7, see chapter 2) was originally proposed for purification of enterocin B from *E. faecium* BFE 900. However, this medium was found to be unsuitable in several purification trials by methods similar to those described above (results not shown), because contaminating medium proteins at the HPLC stage interfered with successful purification. Purification of enterocin B from APT broth, however, proved successful. *E. faecium* BFE 900 was originally isolated from olives (Chapter 2), and in this study it is shown that it produces a bacteriocin identical to enterocin B from *E. faecium* T136 that was isolated from meat (Casaus et al., 1997). The EntB prepeptides of *E. faecium* strains T136 and BFE 900 are also produced with identical leader peptides. As mentioned by Casaus et al. (1997) enterocin B has strong sequence similarity to carnobacteriocin A (CbnA) produced by *C. piscicola* LV17A (Worobo et al., 1994). Both consist of 53 amino acids and they have 47% identical amino acids. Their leader peptides both contain 18 amino acids and share 72% identity (Casaus et al., 1997). Comparison of the mature EntB and CbnA peptides by Lipman-Pearson protein alignment indicates 58.5% similarity. Although the N-terminal extension of EntB

has homology to leader sequences of other bacteriocins, the mature bacteriocin does not have homology to other bacteriocins, except CbnA (Casaus et al., 1997).

The mass of enterocin B determined by electrospray mass spectrometry was 2 Da. less than the theoretically calculated mass, indicating that a disulfide bridge is probably formed between the two cysteine residues at positions 23 and 52. The molecular mass of EntB determined in this study is identical to that reported by Casaus et al. (1997). A second peak with a molecular mass of 5479.0 Da. was detected by electrospray mass spectrometry, which was similar to the masses of enterocin B determined by MALDI-TOF mass spectrometry. This peak was 16 Da. more than the enterocin B peak and probably represents enterocin B with an oxidized methionine residue. The peaks obtained for enterocins A and B in MALDI-TOF mass spectrometry differed from the molecular masses reported for these bacteriocins (Aymerich et al., 1996; Casaus et al., 1997) by several Da. This could be explained by the fact that internal calibrants were not used together with the culture supernatants in MALDI-TOF mass spectrometry. Theoretically the use of such internal calibrants should result in more accurate mass determinations. However they may interfere with mass spectometry of compounds that are present in low amounts in the culture supernatant by supressing analyte ion formation, leading to detection of only the calibrant peaks.

Three base pairs upstream of the –35 (1) site of one of the two putative promoter sequences for *entB* is a conserved regulatory like box. Diep et al. (1996) suggested that such sequences are conserved in 'regulated' bacteriocins. The conserved regulatory-like box upstream of the enterocin B promoter region suggests that production of EntB is regulated and that it could rely on the gene for an induction factor (see below) as well as an unidentified response regulator (RR) and a protein histidine kinase (HK). A conserved regulatory-like box sequence was not observed upstream of the *entB* gene in *E. faecium* T136 (Casaus et al., 1997). This is surprising because the nucleotide sequence reported for the *entB* gene by Casaus et al. (1997) is identical to the sequence shown in Fig. 3.3. However, the nucleotide sequence upstream of the RBS of *entB* in this study differs from that reported by Casaus et al. (1997).

The putative protein product of *orf6* in this study has the characteristics of an induction factor (IF), because it contains a double glycine-type leader peptide and the

mature peptide is relatively small in size (21 amino acids). Similar to other IF genes a conserved regulatory-like box (9 bp repeat spaced by 13 nucleotides) is located upstream of *orf6*. This putative IF differs from the IF previously described to be responsible for induction of enterocins A and B (Nilsen et al., 1998), which was named EntF. EntF consists of a 25 amino acid prepeptide, which bears a 16 amino acid double-glycine-type leader peptide (Nilsen et al., 1998). In contrast to other IF genes for bacteriocins, no typical conserved regulatory-like box sequence was found upstream of *entF*, but a 9 bp repeat separated by 25 nucleotides was detected (Nilsen et al., 1998).

Using MALDI-TOF mass spectrometry it was observed that production of enterocin B in *E. faecium* BFE 900 was constitutive, whereas production of enterocin B in *E. faecium* CTC492 was induced. Constitutive production of enterocin B in strain BFE 900 suggests that the product of *orf6*, which has the characteristics of an induction factor, is probably not associated with bacteriocin production. Speculatively, it is possible that enterocin B production in strain BFE 900 was regulated at a past time, and that during the course of evolution the bacteriocin may have become constitutively produced as a result of a mutational event. The presence of a conserved, regulatory-like box upstream of the enterocin B structural gene supports the hypothesis of regulated bacteriocin production. Alternatively, EntF could be responsible for regulation of bacteriocin production by strain BFE 900 similar to the case of strain CTC492 reported by Nilsen et al. (1998).

Transcription regulators control their target operon by binding directly to inverted or direct repeats which are located close to the promoter region. This binding stimulates binding of the RNA polymerase to the promoter –35 and –10 sites (Diep et al., 1996; Nes et al., 1996). The presence of a controlled regulatory-like box upstream of *entB* implies that for efficient transcription of the gene a promoter regulation factor is required for this interaction with the promoter to occur. Absence of such a regulatory factor could mean that transcription would be inefficient. If a transcription regulator is absent the question is how enterocin B is still efficiently produced? Clearly, production occurs efficiently as determined experimentally by antimicrobial activity assays and mass spectrometry. One possible solution to this paradox is that a second putative set of –10 and –35 promoter sequences was identified upstream of *entB*. These promoter sequences are conserved to the same degree as the first set of –10 and –35 promoter sequences, but they are located

closer to the EntB structural gene (Fig. 3.3). Speculatively, these promoter sequences would not require the interaction with a transcriptional regulator and could ensure efficient constitutive production of bacteriocin.

Deferred inhibition tests with *E. faecium* BFE 900 and an EntB$^+$ *E. faecalis* clone against various indicators suggested that the wild-type strain produced multiple bacteriocins. We obtained a PCR amplicon that coded for part of the mature enterocin A gene identical to that produced by *E. faecium* T136 (Casaus et al., 1997) and *E. faecium* CTC492 (Aymerich et al., 1996). In addition, results from MALDI-TOF mass spectrometry indicated that both enterocins A and B are produced by *E. faecium* BFE 900. Therefore, in a further hypothetical scenario, enterocin B production in strain BFE 900 could be regulated by the enterocin A compound. Our MALDI-TOF data suggest that enterocin A is constitutively produced, thus a constant supply of enterocin A could ensure constant induction of enterocin B production. Induction of bacteriocin production by a bacteriocin compound *per se* was demonstrated for carnobacteriocin B2. Both this carnobacteriocin B2 and an induction factor CbnS were shown to be capable of inducing B2 production (Quadri et al., 1997). However, production of enterocins A and B in strain CTC492 is lost upon dilution of the culture to extinction (Nilsen et al., 1998), suggesting that EntF is the induction factor for enterocin B in that strain. Further experimentation is required to elucidate the basis for constitutive and regulated production of enterocin B in *E. faecium* strains BFE 900 and CTC 492.

EntB differs from other class IIa bacteriocins because it does not contain a YGNGVXC– consensus motif near the N-terminus (Klaenhammer, 1993), but similar to class IIa bacteriocins it has a double-glycine-type leader peptide that is associated with a dedicated bacteriocin transport system (Nes et al., 1996). Heterologous expression could not be attempted with EntB transporter proteins, because the genes for EntB-dedicated ABC transporter or accessory transport proteins were not found on either the 2.2-kb or 12.0-kb cloned chromosomal fragments. This could mean that secretion of EntB depends on the bacteriocin-dedicated transport proteins of a different bacteriocin, such as enterocin A. The fact that secretion of carnobacteriocin BM1 by *C. piscicola* is dependent on bacteriocin-dedicated transport proteins associated with carnobacteriocin B2 production (Quadri et al., 1994) supports this hypothesis.

For most class II bacteriocins described to date, the structural gene is in an operon with an immunity gene located adjacent to and downstream of the structural gene (Klaenhammer, 1993, Nes et al., 1996). This was not the case for EntB; it resembles the case of CbnA, in which an immunity gene also did not occur in the same operon as the bacteriocin structural gene. However, the immunity gene for enterocin B was located in the opposite orientation immediately downstream of the EntB structural gene, which is different to the CbnA case where an immunity gene could not be located in the immediate vicinity of the bacteriocin structural gene. This is the first report of an immunity gene for the class IIa bacteriocins that does not contain the N-terminal YGNGVXC box sequence but shares sequence homology at the C-terminus.

The 12.0-kb *Eco*RI chromosomal DNA fragment of *E. faecium* BFE 900 contained genes for a phospho-β-glucosidase, a transport protein (enzyme II^{Bgl}) that is a member of the phosphoenolpyruvate-dependent carbohydrate-phosphotransferase system and a transcription antiterminator protein, but apart from the EntB structural and immunity genes, no other genes associated with bacteriocin production were unequivocally identified. This is in contrast to the presence and arrangement of genes in bacteriocin loci of other bacteriocin-producing bacteria, including the similar bacteriocin carnobacteriocin A, in which gene clustering is often reported for genes involved in bacteriocin production and regulation (Klaenhammer, 1993; van Belkum and Stiles, 1995; Nes et al., 1996; Brurberg et al., 1997, Quadri et al., 1997). The case of isolated enterocin B structural and immunity genes thus is rare, and is only paralleled by the case of carnobacteriocin BM1, where the similarly isolated structural and immunity genes are located on the chromosome of *C. piscicola* LV17 (Quadri et al., 1994). In contrast to the case of CbnBM1, the EntB bacteriocin structural and immunity genes are not arranged in an operon and this represents a unique case.

The leader peptide of EntB is similar to that of CbnA, with 13 of 18 amino acids identical, which prompted investigation of the heterologous expression of EntB using the CbnA transport proteins. Cloning of the EntB structural gene into *C. piscicola* LV17A resulted in activity of this strain against *L. lactis* ATCC 19435, an EntB sensitive, CbnA resistant indicator. This suggested that the enterocin leader peptide was recognized by the

ABC transporter for CbnA. Heterologous expression of enterocin B was also achieved by the *sec*-pathway. The zone of antimicrobial activity observed for the wild type was larger than that observed for the heterologous host. This could be explained by the fact that the wild-type produces additional bacteriocin (such as enterocin A). This would be in accordance with earlier results (Casaus et al., 1997) that showed that enterocin A and enterocin B act synergistically.

In this study we took advantage of the homology between leader peptides of enterocin B and carnobacteriocin A to obtain heterologous expression of enterocin B by the carnobacteriocin A dedicated secretion pathway. The homology observed between the mature bacteriocins also suggests a relationship between structure and biological function (Casaus et al., 1997). An interesting and unanswered question is whether the homology observed for these bacteriocins is also reflected by homology in their respective immunity proteins. The gene encoding an immunity protein for carnobacteriocin A has not been determined. However, it is known that similar to the case of the enterocin B immunity gene, the gene for carnobacteriocin A immunity is not located in an operon together with the bacteriocin gene. Chapter 4 deals with the localization of the carnobacteriocin A immunity gene, as well as comparisons between the genetics of enterocin B and carnobacteriocin A immunity.

3.5. References

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CHAPTER 4

IDENTIFICATION OF THE GENE ENCODING IMMUNITY TO CARNOBACTERIOCIN A IN *Carnobacterium piscicola* LV17A.

4.1. Introduction.

Bacteriocins produced by LAB are compounds that have antimicrobial activity against closely related strains. In some cases they can also be active against gram-positive foodborne pathogens such as *Listeria monocytogenes, Staphylococcus aureus,* and *Clostridium botulinum* spores, and for this reason these compounds have been suggested for biopreservation in foods (Klaenhammer, 1993; Abee et al., 1995; Jack et al., 1995, Stiles, 1996). Class II bacteriocins are small (<10 kDa), heat stable, non-lanthionine containing compounds that act on the membrane of sensitive cells (Klaenhammer, 1993). They form pores in sensitive membranes, causing efflux of small intracellular compounds such as amino acids and potassium ions. In addition, they dissipate the proton motive force which inhibits amino acid transport (Chikindas et al., 1993; Abee, 1995; Jack et al., 1995).

A general model for pore formation by class II bacteriocins involves binding of the bacteriocin to a receptor followed by insertion into the membrane. Bacteriocin monomers aggregate in the membrane with hydrophobic sides facing the membrane and hydrophilic sides forming the pore (Klaenhammer, 1993; Abee et al., 1995, Jack et al., 1995). Evidence for the involvement of a receptor for bacteriocin binding has been obtained for some bacteriocins, e.g., lactococcin A and pediocin PA-1 (Chikindas et al., 1993; Venema et al., 1994). However, insertion of other bacteriocins, e.g., thermophilin 13 and plantaricin C (González et al., 1996; Marciset et al., 1997), into the membrane appears to be independent of a receptor.

Class II bacteriocin-producing LAB have an 'immunity system' to protect the cell from it's own bacteriocin. Such immunity depends on production of an immunity protein. Each bacteriocin has its own dedicated immunity protein that is expressed concomitantly with the bacteriocin (Jack et al., 1995; Nes et al., 1996). In the case of multiple

bacteriocin production, a specific immunity protein is produced that is responsible for self-protection against each of the bacteriocins. For example, *L. lactis* subsp. *cremoris* 9B4 produces three different bacteriocins (lactococcins A, B and M) and three corresponding specific immunity proteins (van Belkum et al., 1991, 1992). In most cases, the bacteriocin immunity gene is located on the same transcription unit next to and downstream of the bacteriocin structural gene (Nes et al., 1996).

Immunity proteins may have different ways to protect the producer cell from the lethal effect of a bacteriocin. For example, the immunity protein for carnobacteriocin B2 was suggested to block the pore formed by bacteriocins from the cytoplasmic side. This suggestion was based on the fact that no transmembrane segments could be predicted for the carnobacteriocin B2 immunity protein, indicating that it could not associate with the membrane. In agreement with this hypothesis, most of the immunity protein was localized in the cytoplasm (Quadri et al., 1995). Other immunity proteins, e.g., LafI and LciM (van Belkum et al., 1991; Allison and Klaenhammer, 1996) were predicted to have transmembrane helices. An amphiphilic α-helical domain (residues 29 to 47) of LciA was suggested to be embedded in the membrane (Venema et al., 1994). Given the possible membrane associations of LafI, LciM and LciA, these immunity proteins were suggested to interact with the receptor for the bacteriocin and to prevent bacteriocin binding to the membrane of the producer cell (Venema et al., 1994; Allison and Klaenhammer, 1996).

Despite the similarities observed for many class II bacteriocins, their immunity proteins share little homology (Allison and Klaenhammer, 1996; Aymerich et al., 1996; Nes et al., 1996). This lack of similarity is exemplified in the case of the immunity proteins of the two identical bacteriocins curvacin A and and sakacin A, where the putative immunity proteins are 51 and 90 amino acids in size, respectively (Axelsson et al., 1993; Tichaczek et al., 1993). The only common characteristics between immunity proteins are that they are cationic (pI ranging from 7.3 to 10.3) and relatively small (generally less than 1 kDa) (see chapter 1, Table 1.3; Allison and Klaenhammer, 1996; Diep et al., 1996).

Carnobacterium piscicola LV17 produces three bacteriocins, carnobacteriocins A, BM1 and B2. The structural gene for carnobacteriocin BM1 (CbnBM1) is located on the chromosome and those for carnobacteriocin A (CbnA) and B2 (CbnB2) are located

on a 72-kb plasmid pCP49 and a 61-kb plasmid pCP40, respectively (Quadri et al., 1994; Worobo et al., 1994). By curing and plasmid mobilization experiments the plasmids were separated, and introduced into the plasmidless host strain (LV17C) as LV17A and LV17B containing the 72 and 61-kb plasmids, respectively (Ahn and Stiles, 1992). Although the immunity genes for CbnBM1 and CbnB2 were located within the same operon with the bacteriocin structural genes (Quadri et al., 1994), no immunity gene could be located with the bacteriocin structural gene for CbnA (Worobo et al., 1994). This is similar to the case for enterocin B (see chapter 3). Carnobacteriocin A and enterocin B are 47% identical at the amino acid level, and both are different from other class IIa, 'pediocin-like' bacteriocins in that they do not contain the YGNGVXC consensus motif at the N-terminus of the mature peptide (see chapter 3). These similarities prompted us to localize the immunity gene for CbnA, and to determine whether this gene encodes an immunity protein similar to the enterocin B immunity protein.

4.2. Methods and materials.

4.2.1. *Bacterial strains and culture conditions.*

The bacterial strains and plasmids used in this study are shown in Table 4.1. The carnobacteriocin A producer *Carnobacterium piscicola* LV17A and the carnobacteriocin A sensitive, plasmidless *C. piscicola* LV17C strains were grown in APT broth (Difco Laboratories Inc., Detroit, Michigan) at 25°C. *E. coli* strains were grown on a rotary shaker at 250 rpm in Luria Bertani broth (LB; Becton Dickinson, Cockeysville, Maryland) at 37°C. All cultures were incubated aerobically. Antibiotics were added as selective agents when appropriate: erythromycin (200 µg/ml) and ampicillin (150 µg/ml) for *E. coli*, and erythromycin at 5 µg/ml for LAB strains. Stock cultures were made in the

Table 4.1 Bacterial strains and plasmids used in this study.

Bacterial Strain or Plasmid	Relevant Characteristics	Reference or Source
Strains		
C. piscicola LV17A	*cbnA*$^+$*cbi*A$^+$ containing pCP49	Ahn and Stiles (1990), Worobo et al. (1994)
Carnobacterium piscicola LV17C	CbnA^S, plasmidless	Ahn and Stiles (1990)
Lactobacillus sake DSM 20017	EntB^S	
Escherichia coli DH5α	F$^-$ *endA1 hsdR17* (r_k-m_k+) *supE44*thi-1 λ^- *recA1 gyrA96 relA1 Δ (argF-lacZYA)* U169 Φ80*dlacZΔM15*	BRL Life Technologies Inc.
E. coli MH1	MC1061 derivative; *ara*D139 *lacX74 galU galK hsr hsm*$^+$ *strA*	Casadaban and Cohen (1980)
Plasmids		
pUC118	*lacZ'* Amp^r, 3.2 kb	Vieira and Messing (1987)
pMG36e	Expression vector, Em^r, 3.6-kb	Van de Guchte et al. (1989)
pGKV259	Expression vector, Em^r,Cm^r, 5.0-kb	Van der Vossen et al. (1987)
pCMAP07	pMG36e containing 5.4-kb *Sma*I fragment from pRW9.6 (Worobo, 1996)	This study
pCMAP08	pMG36e containing 3.1-kb *Pst*I-*Sph*I fragment from pCMAP07	This study
pCMAP09	pMG36e containing 1.9-kb *Bsp*MI-*Sph*I fragment from pCMAP08	This study
pCMAP10	pMG36e containing 1.4-kb *Bam*HI-*Sph*I fragment from pCMAP08	This study
pCMAP11	pMG36e containing 751-bp *Cla*I-*Sph*I fragment from pCMAP08	This study
pCMAP12	pMG36e containing 721-bp *Bam*HI-*Cla*I fragment from pCMAP08	This study
pCMAP13	pMG36e containing 400-bp *Bam*HI-HindIII fragment from pCMAP08	This study
pCMAP14	pMG36e containing 231-bp *Xba*I-*Kpn*I PCR product insert of *cbiA*	This study
pCMAP15	pGKV259 containing *Sal*I-*Pst*I PCR product insert of *cbiA*	This study

same medium used for culturing the bacterial strain with 15% glycerol (vol./vol.), and were stored at -80°C.

4.2.2. *Deferred inhibition tests and bacteriocin activity assays.*

Deferred inhibition tests were carried out by the methods of Ahn and Stiles (1990), and producer cultures were spotted onto APT agar. Bacteriocin activity assays were done by the critical dilution assay as described in chapters 2 and 3. Indicator bacteria were inoculated (1%) into the appropriate soft (0.75%) agar medium. *Carnobacterium piscicola* LV17C was used as the carnobacteriocin A sensitive indicator strain.

4.2.3. *DNA isolation, manipulation and electroporation.*

Small and large scale DNA isolations from *E. coli* were done according to Sambrook et al. (1989), and from LAB according to van Belkum and Stiles (1995) and Worobo et al. (1994). DNA fragments were recovered from agarose gels using QIAEX II (QIAGEN, Chatsworth, California). Restriction enzymes and T4 DNA ligase (New England Biolabs, Missisauga, Ontario, Canada) and Klenow enzyme (Promega, Madison, Wisconsin) were used as recommended by the suppliers. Cloning and DNA manipulations were done as described by Sambrook et al. (1989). Competent cells of *E. coli* were prepared and transformed according to the one-step method of Chung et al. (1989). Recombinant pMG36e plasmids were first transformed into *E. coli* MH1 before being transformed into LAB. Carnobacteria were transformed by electroporation according to Worobo et al. (1995).

4.2.4. *Localization of the carnobacteriocin A immunity gene.*

A 9.6-kb *Pst*I fragment from pCP49 was previously cloned into the *Lactobacillus* cloning vector pCaT (Jewell and Collins-Thompson, 1989). This fragment was sequenced in both directions, and it was shown to contain all of the genes necessary for bacteriocin production and immunity (Worobo, 1996). A 5.4-kb *Xba*I-*Pst*I fragment was derived from this fragment and it was also cloned into pCaT; this 5.4-kb fragment failed to produce carnobacteriocin, but it did confer full immunity to carnobacteriocin A (Worobo, 1996). In the search for the carnobacteriocin A immunity gene, this fragment was excised from pCaT, blunt ended and cloned into the *Sma*I site of the lactococcal shuttle vector pMG36e in correct orientation to the P32 promoter (van de Guchte et al., 1989), resulting in plasmid pCMAP07. A 3.1-kb *Pst*I-*Sph*I fragment was also excised from the 5.4 fragment in pCaT and cloned into pMG36e, resulting in plasmid pCMAP08. This 3.1-kb fragment was progressively shortened by cutting plasmids at the *Pst*I site of pMG36e, and at unique restriction enzyme sites within the fragment (Fig. 4.1). After the restriction enzyme digests, the plasmids were blunt ended, and self-ligated. Hence, plasmids pCMAP09, 10, 11, 12, and 13 contained various regions of the 3.1-kb fragment in pCMAP08 (Figure 4.1).

4.2.5. *PCR amplification and cloning of the carnobacteriocin A immunity gene.*

The carnobacteriocin A immunity gene (*cbiA*) was amplified by PCR using plasmid pCMAP08 as template. Primers CFR-08 (5'-TAT ATC TAG AGA TCT AAT CAA AAT AAC TAG GA-3') and CFR-09 (5'-TAT AGG TAC CGT CTA CAG TCT GAA ACT AAA A-3') were complementary to the 5' and 3' ends of this gene in pCMAP08, and also contained *Xba*I and *Kpn*I restriction sites.

In this PCR reaction DNA was amplified in a 100 µl volume using a temperature cycler (Omnigene). The PCR mixture contained 1.0 µM of each of the respective primers, 200 µM of dNTPs, 1 unit of *TaqPlus*™ Precision DNA polymerase, and 1x *TaqPlus* Precision buffer (Stratagene, La Jolla, California). DNA was amplified in 32 cycles (denaturation, 94°C, 1 min; annealing, 52°C, 1 min; extension, 72°C, 1 min).

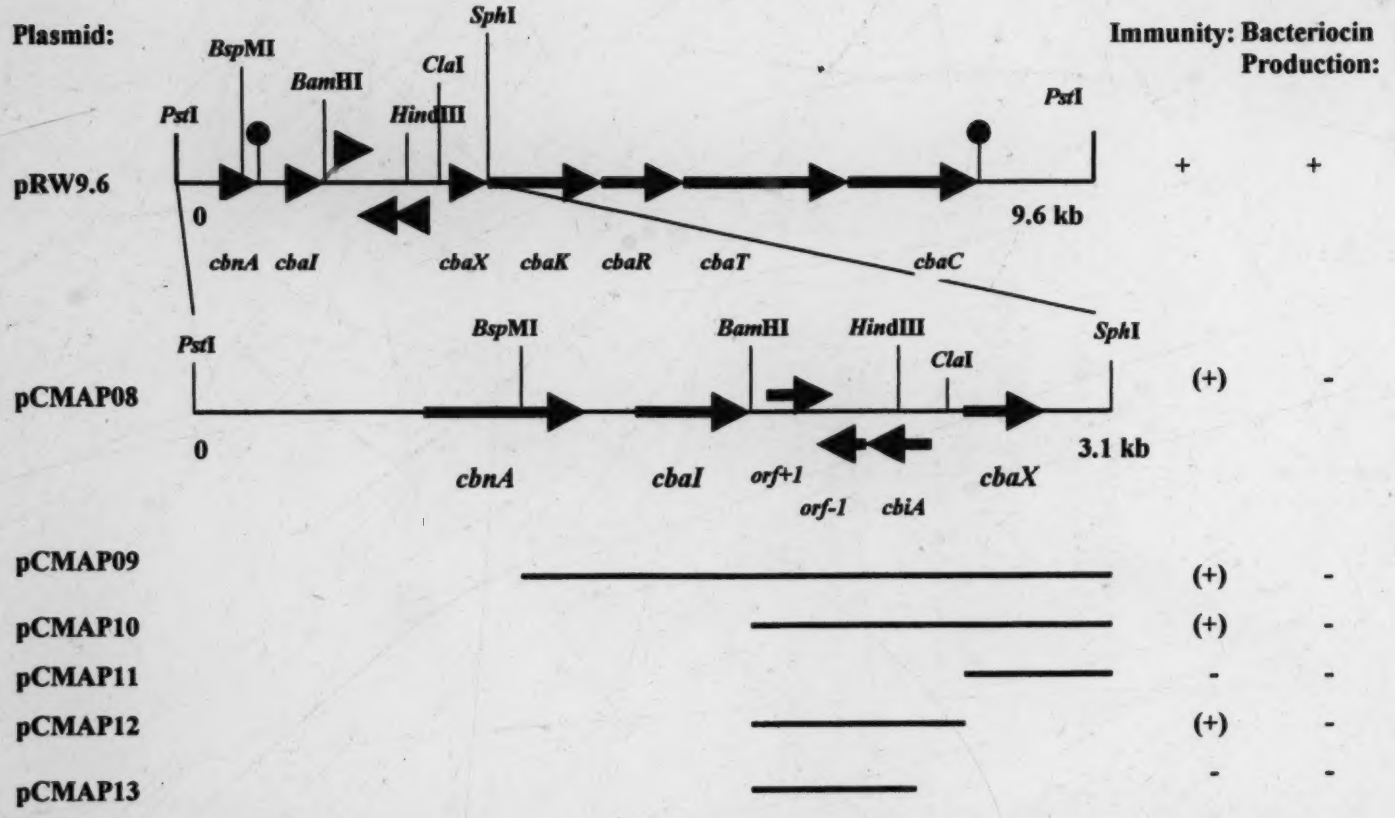

Figure 4.1 Organization of genes involved in carnobacteriocin A production on plasmids pRW9.6 and pCMAP08, and schematic presentation of gene fragments cloned behind the P32 promoter in pCMAP plasmids. Bacteriocin activity and immunity of the constructs when cloned into *C. piscicola* LV17C is shown, (+) denotes partial immunity. Stem-loop vertical lines represent transcriptional terminators.

The PCR product was cloned into pUC118 for sequencing to confirm the fidelity of the reactions.

Plasmid pCMAP14 was created by cloning the carnobacteriocin A immunity gene PCR product into the *Xba*I-*Pst*I sites of pMG36e under the control of the P32 promoter (van de Guchte et al., 1989). The *cbiA* gene was also excised from this plasmid and cloned into the related lactococcal shuttle vector pGKV259 under the control of the P59 promoter, resulting in plasmid pCMAP15. The P59 promoter is also a lactococcal promoter but it is stronger than the P32 promoter (van der Vossen et al., 1987).

4.2.6. *Homologous expression of carnobacteriocin A immunity.*

For homologous expression of immunity, plasmids pCMAP07 to 15 were used to transform *C. piscicola* LV17C, and transformants were used as indicators in deferred inhibition tests with *C. piscicola* LV17A containing pMG36e as the producer strain. All constructs cloned into pMG36e were under the control of the P32 lactococcal promoter (van de Guchte et al., 1989), while the *cbiA* gene in pCMAP15 was under the control of the P59 promoter. Presence of plasmid DNA in transformants was confirmed by small scale plasmid isolations.

4.3. Results.

4.3.1. Identification of the carnobacteriocin A immunity gene.

Several genes associated with carnobacteriocin A production were identified on the 9.6-kb fragment of pCP49 (Worobo, 1996) (Figure 4.1). These included the carnobacteriocin A structural gene (*cbnA*), a putative immunity gene (*cbaI*), genes with homology to dedicated bacteriocin transporter proteins, i.e., an ATP binding cassette (ABC) transporter (*cbaT*) and an accessory gene (*cbaC*), as well as genes with homology to three component regulatory systems, i.e., a putative induction factor gene (*cbaX*), a histidine kinase gene (*cbaK*) and a response regulator gene (*cbaR*) (Worobo, 1996). The 5.4-kb fragment cloned into pCaT was missing *cbaT* and *cbaC* and failed to

produce bacteriocin (Worobo, 1996). However, when this fragment was cloned into pCaT as well as pCMAP07 it conferred full immunity to *C. piscicola* UAL26 (Worobo, 1996) and *C. piscicola* LV17C (this study), respectively (Figures 4.1 and 4.2).

Interestingly, the 3.1-kb *Pst*I-*Sph*I fragment in pCMAP08 conferred only partial immunity to the *C. piscicola* LV17C host, as indicated by a smaller zone of activity when tested against the *C. piscicola* LV17A producer in the deferred inhibition test. This zone was similar to that shown for partial inhibition of *C. piscicola* LV17C containing pCF14 in Figure 4.2. This fragment differed from the 5.4-kb fragment in that the response regulator gene and part of the histidine kinase gene were not present (Figure 4.1). Plasmid pCMAP09 which contained the proposed carnobacteriocin immunity gene *cbaI* (Worobo, 1996) conferred partial immunity to *C. piscicola* LV17A. However, plasmid pCMAP10 did not contain *cbaI*, but this plasmid still imparted partial immunity to *C. piscicola* LV17C in deferred inhibition assays. This indicated that the protein product of *cbaI* (Worobo, 1996) was not associated with immunity to carnobacteriocin A.

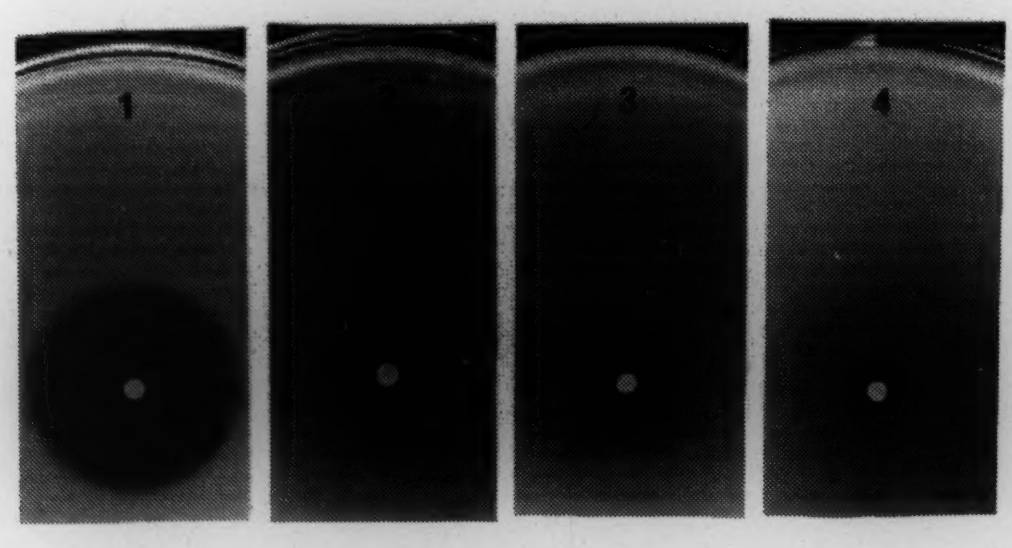

Figure 4.2. Deferred inhibition tests with *Carnobacterium piscicola* LV17A containing pMG36e against *C. piscicola* LV17C containing pMG36e (1), pCF14 (2), pCF15 (3) and pCMAP07 (4).

The carnobacteriocin A immunity gene was present on a 721-bp *Bam*HI-*Cla*I fragment in pCMAP12 (Figure 4.1) because this construct conferred partial immunity to *C. piscicola* LV17C in deferred inhibition assays. Three open reading frames were identified on this fragment, one (*orf+1*) orientated in the 5' to 3' direction and two (*orf-1* and *cbiA*) oriented in the opposite direction (Figure 4.1). *CbiA* contained a *Hin*dIII site, which was used to disrupt this ORF in pCMAP13. Because partial immunity did not result when this plasmid was transformed into *C. piscicola* LV17C, *cbiA* was identified as the carnobacteriocin A immunity gene. *CbiA* was amplified by PCR and cloned into the pMG36e expression vector. *CbiA* conferred the same partial immunity as observed for *C. piscicola* LV17C containing pCMAP08, 09, 10 or 12. This ORF was absent in pCMAP11 and *C. piscicola* LV17C carrying this plasmid did not exhibit the partial immunity phenotype in deferred inhibition assays.

4.3.2. *Effect of promoter strength on homologous expression of immunity.*

Partial immunity was also observed when the *cbiA* gene was cloned behind the P59 promoter in pCMAP15, and expressed in the *C. piscicola* LV17C homologous host. However, the zone of activity in the deferred inhibition test was smaller than the zone of inhibition observed when partial immunity resulted from *cbiA* expressed under the control of the P32 promoter in pCMAP14 (Figure 4.2).

4.4. Discussion.

Immunity genes for class II bacteriocins generally occur close to and downstream of the bacteriocin structural gene, and both genes are usually transcribed as a unit (Klaenhammer, 1993; Nes et al., 1996). This was not the case for carnobacteriocin A, because the immunity gene was not located in an operon together with the *cbnA* gene (Worobo et al., 1994; Worobo, 1996). This resembled the case of enterocin B, where the immunity gene was also not arranged in an operon together with the enterocin B structural gene (see chapter 3). Both enterocin B and carnobacteriocin A are heat stable, small bacteriocins which do not contain lanthionine and are active against

Listeria monocytogenes (Worobo et al., 1994; chapter 3). These characteristics allow grouping of these two bacteriocins in class IIa (Klaenhammer, 1993; Nes et al., 1996); however, enterocin B and carnobacteriocin A differ from other class IIa bacteriocins because they do not contain the YGNGVXC consensus motif at their N-termini. In addition, the separation of bacteriocin structural genes and immunity genes reported in chapter 3 and in this study also differentiates these bacteriocins from other class IIa bacteriocins.

The immunity gene for enterocin B was located immediately downstream of and in opposite orientation to the bacteriocin structural gene (chapter 3). This was different for the carnobacteriocin A immunity gene because an ORF that could encode an immunity protein was not detected close to the bacteriocin structural gene (Worobo, 1996). The small (153 nucleotides) ORF *cbaI* located 255 bases downstream of the carnobacteriocin A structural gene was previously postulated to encode the carnobacteriocin A immunity protein. When a 700-bp *Stu*I-*Hin*dIII fragment containing this ORF was cloned and used in heterologous expression tests, immunity to carnobacteriocin A was not observed (Worobo, 1996). It was speculated that this potential immunity gene may be regulated, and that it was possibly controlled by the response regulator gene (*cbaR*). Failure to detect immunity thus could be explained by the fact that gene required the presence of the response regulator gene for promotion of transcription (Worobo, 1996). Results of this study suggested that *cbaI* was not the carnobacteriocin A immunity gene, because absence of this gene in pCMAP10 did not diminish the partial immunity observed.

By making sequential deletions of the 3.1-kb fragment in pCMAP08 the gene conferring partial immunity to CbnA was identified. When this gene was amplified by PCR and cloned into *C. piscicola* LV17C, it also conferred partial immunity to this homologous host. It is not clear why this gene did not confer full immunity to this host, but a partial immunity phenotype has also been observed in other bacteriocin systems. In class I bacteriocin producing strains, full immunity to the bacteriocin may be dependent on the presence of several genes. For example, immunity to nisin produced by *Lactococcus lactis* subsp. *lactis* strains depends on the presence of the *nisI* immunity gene, which is under common regulation with the *nisA* structural gene (Kuipers et al.,

1993), but the *nisE*, *nisF* and *nisG* genes are required for full immunity (Siegers and Entian, 1995). NisE and NisF show high homology to ABC transporter proteins, whereas NisG encodes a hydrophobic protein that may act in a similar manner to the immunity proteins described for several colicins (Garrido et al., 1988; Siegers and Entian, 1995). The ABC transporter protein involved with transport of lacticin 481 in *Lactococcus lactis* strains is assembled from 3 subunits that are encoded by the genes *lctF*, *lctE* and *lctG*. The presence of these three genes was shown to be sufficient for full immunity to lacticin 84 (Rincé et al., 1997). The cyclic peptide bacteriocin AS-48 produced by *E. faecalis* S-48 requires the presence of the *as-48D1* gene for immunity. The protein encoded by this gene is cationic and it was suggested to be membrane associated (Martínez-Bueno et al., 1998). In addition, the presence of the genes *as-48B*, *as-48C1* and *as-48D* are required for full immunity. These genes were suggested to encode subunits of an ABC transporter (*as-48C1* and *as-48D*) as well as a protein involved with bacteriocin maturation (*as-48B*) (Martínez-Bueno et al., 1998). Therefore, ABC transporter proteins may also have an immunity function in some bacteriocin systems. However, in the case of carnobacteriocin A immunity the association of transport proteins with immunity was considered unlikely. Only the N-terminal part of the putative ABC transporter of CbnA was present and the putative CbnA accessory protein gene was absent in plasmid pCMAP07. Yet in homologous expression experiments this plasmid conferred full immunity to CbnA.

Interestingly, the switch from a fully immune to a partially immune phenotype only occurred in the absence of functional CbnA histidine kinase and response regulator genes. This suggests that production of carnobacteriocin A immunity, as previously suggested by Worobo (1996), is regulated. This suggestion is supported by similar observations that the absence of regulatory genes decreases immunity. For example, the presence of the putative response regulator gene *nisR* was shown to be required for nisin production and this gene was also shown to regulate nisin immunity (van der Meer et al., 1993). For the carnobacteriocin B2 cluster, it was shown that inactivation of the response regulator resulted in loss of immunity (Quadri et al., 1997).

If the carnobacteriocin A immunity gene is regulated, stronger promoter activity should increase the observed level of immunity. To test this possibility the *cbiA* gene was cloned behind the P59 promoter in pCMAP15. This is a stronger promoter than the P32

promoter present in the pCMAP08, 09, 10, and 12 plasmids that conferred partial immunity to carnobacteriocin A. When pCMAP15 was cloned into the CbnA sensitive *C. piscicola* LV17C strain, an increased level of immunity was observed in deferred inhibition tests, based on the size of the inhibition zone. This suggests that promoter activity could account for varying levels of immunity. It is not known why full immunity was not obtained by using plasmid pCMAP15 in homologous expression experiments. However, differences in strength of the native regulated promoter for *cbiA* and the P59 promoter, as well as gene dosage effects based on different copy numbers of the pCP49 and the pGKV259-based plasmids may explain the differences between full and partial immunity.

Similar to the enterocin B immunity gene, the carnobacteriocin A immunity gene is located downstream and in opposite orientation to the bacteriocin structural gene. CbiA consists of 56 amino acids which is two amino acids smaller than EniB (58 amino acids); the proteins are very alike and comparison by Lipman Pearson protein alignment indicates 64.3% similarity. Similar to the enterocin B immunity protein, CbiA is hydrophobic and contains charged amino acids at the amino and carboxy ends. Using the PepTool protein structure prediction software (chapter 3) the protein was also predicted to form an α-helix that may insert into the membrane. The similarities observed for both of the mature bacteriocins enterocin B and carnobacteriocin A and their respective immunity genes raises the question whether the EniB and the CbiA proteins can impart cross-protection to their respective bacteriocin counterparts and warrants further investigation.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS.

The lactic acid bacteria are a heterogeneous group of microorganisms that are non-sporeforming, catalase-negative and produce lactic acid as the major end product of fermentation. These bacteria have been used in the production of fermented foods since ancient times and, in modern times, production of fermented dairy, meat, vegetable and cereal products depends on the use of defined LAB starter cultures (Stiles and Holzapfel, 1997). Fermented foods constitute a quarter of the western diet and are characterized by a safe history, improved organoleptic properties and extended shelf life (Lindgren and Dobrogosz, 1990; Hammes and Tichaczek, 1994). Because of the long association of some of the LAB with food fermentation they are designated as generally recognized as safe (GRAS) organisms.

Enterococci are LAB that have also been used to advantage in the food industry. Enterococcal strains are used for the production of certain types of cheeses in Southern European countries (described in chapter one), and certain enterococcal strains have been used as probiotics as an alternative to antibiotic treatment for gastroenteritis in humans (Lewenstein et al., 1979; Bellomo et al., 1980; Bruno and Frigerio, 1981). Enterococci are ubiquitous in the environment and also occur in the gastrointestinal tracts of animals such as cattle and poultry, as well as humans (Devriese and Pot, 1995). As a result of their gastrointestinal origin, the enterococci have been used as indicator bacteria for fecal contamination of waste water (Leclerc et al., 1996). The enterococci are hardy bacteria which exhibit a high heat resistance and survive under adverse environmental conditions. For these reasons they have frequently been proposed as indicators of sanitary quality of foods (Jay, 1996). The fecal origin of these bacteria leads to a high potential for contamination of flesh-types of food and their hardy nature may allow them to survive in meat or poultry processing environments. Therefore, on meat and poultry products the enterococci may constitute an undesirable part of the adventitious microflora, especially in the case of heat treated products, where these heat resistant bacteria may survive the

heat treatment and cause product spoilage (Houben, 1982; André Gordon and Ahmad, 1991).

An emerging concern for the association of enterococci with foods is that these bacteria, particularly *E. faecalis* and *E. faecium*, have become recognized as major nosocomial pathogens (see chapter 1). The association of these bacteria with human infection can be attributed in part to the fact that these bacteria have become 'multiple antibiotic resistant', and that patients are immunosuppressed for reasons such as steroid or cytotoxic therapy, because of their underlying disease (e.g., malignancy) or because of extremes of age (Morrison et al., 1997). In addition, virulence factors (described in chapter 1) must be involved for these bacteria to cause infection. Virulence factors, as well as antibiotic resistance determinants are often located on plasmids, some of which respond to sex pheromones and transfer at a high frequency (Clewell, 1990).

The implications of pathogenic enterococci in the food supply are not clear. Certainly the enterococci should not be regarded in the same way as other foodborne pathogens, and the incidence of enterococci in human disease does not appear to correlate with the incidence of these bacteria in foods. However, antibiotic resistance genes including those for vancomycin-resistance have been reported in enterococci from foods (Bates et al., 1993; Klare et al., 1995; Teuber et al., 1996). This raises the issue whether food strains may also carry putative virulence factors, such as aggregation substance, hemolysin or gelatinase (see chapter 1). Enterococci are clearly opportunistic pathogens and infections are usually associated with the immunosupressed. However, the food supply should be safe for all, especially the elderly and immunosuppressed.

The food industry should, therefore, take notice of the enterococci and exercise better control over their presence in foods. For example, enterococci should not be allowed to establish themselves as the predominant microflora of heat-treated foods, and care should be exercised when selecting strains of enterococci for use as probiotics or as starter cultures in cheese manufacture. The cultures should be investigated to determine whether the organisms are antibiotic resistant or carry putative virulence factors. The presence of plasmid DNA should be determined because many putative virulence traits and antibiotic resistance genes are plasmid encoded. This is especially important for *E. faecalis* strains that can carry plasmids that respond to sex pheromones and which

transfer at a high frequency. The versatility of enterococci in disseminating genetic material previously led Aguirre and Collins (1993) to question the wisdom of employing enterococci as probiotics. Based on our knowledge of the association of enterococci with pathogenesis in man, it would be difficult to confer GRAS status to enterococcal species for use in foods.

The lactic acid bacteria have long been known to exert their preservative effect in food fermentation by production of antimicrobial compounds, mainly organic acids. However, additional antimicrobial factors such as hydrogen peroxide, diacetyl and bacteriocins may play a part in the preservation of foods (Lindgren and Dobrogosz, 1990; Holzapfel et al., 1995). Bacteriocin production has been documented for most genera of lactic acid bacteria. By definition, bacteriocins are generally active only against closely related strains (Klaenhammer, 1993); however, in some cases gram-positive pathogens such as *Listeria monocytogenes* are also inhibited by LAB bacteriocins. Inhibition of gram-positive spoilage or pathogenic bacteria by bacteriocin-producing LAB has led to suggestions for use of purified bacteriocins or the bacteriocinogenic LAB as biopreservatives in foods (Holzapfel et al., 1995; McMullen and Stiles, 1996; Muriana, 1996; Schillinger et al., 1996). Use of purified bacteriocins in foods would be difficult to achieve because of the relative difficulty of purification and production of these compounds in low amounts (Carolissen-Mackay et al., 1997).

The production of enterococcal bacteriocins (enterocins) with activity against other enterococcal strains would be a possible strategy to control these unwanted bacteria in foods such as meat or poultry products (see above). However, the enterococci may be viewed as potential pathogens (see chapter 1), which makes addition of a bacteriocin-producing starter *Enterococcus* culture to foods an unattractive option. Alternatively, the genetic determinants for enterocins may be cloned into a starter culture that is considered 'food grade'. The 'food grade' status is interpreted as meaning that the starter culture that serves as a heterologous host would be non-pathogenic and come from a food source itself, and that only the genes necessary for bacteriocin production would be cloned into this host. Furthermore, the problem of low bacteriocin yields experienced in enterocin purification may be circumvented by raising antibodies against the enterocin, and using the antibodies in an affinity column. The bacteriocin enterocin B produced by *E. faecium*

BFE 900 has been purified and currently antibodies specific for this bacteriocin are being raised (Rose, 1998). This may allow future rapid purification at high yield to warrant use of the compound as an anti-enterococcal factor in foods.

At the outset of this study bacteriocin production by *Enterococcus* strains had been described, but relatively little was known about the chemical structure of these enterocins and the genes necessary for their production had not been identified. In our search for bacteriocinogenic LAB from vegetable foods we isolated a LAB strain that exhibited antimicrobial activity. This strain was unequivocally identified as *Enterococcus faecium* by using traditional (physiological and carbohydrate fermentation tests) and SDS-PAGE of total soluble cell protein and DNA-DNA hybridization classification techniques. The more modern and specific identification techniques of DNA-DNA hybridization and SDS-PAGE of soluble cell protein were considered crucial in the identification of this isolate as *E. faecium*, because there are no phenotypic criteria available that unequivocally separate the genus *Enterococcus* from the other genera of gram-positive, catalase-negative cocci (Devriese et al., 1993).

The *E. faecium* BFE 900 isolate exhibited a broad antagonistic activity spectrum, and strains of enterococci, *Lactobacillus* spp., *Listeria* spp. including *L. monocytogenes*, *S. aureus* and *Clostridium* spp. were inhibited by using a partially purified culture supernatant of this isolate. The antimicrobial compound was heat stable, inactivated by proteases and had a bactericidal mode of action. This suggested that the antimicrobial compound was a bacteriocin that probably belonged to class IIa bacteriocins, which are defined as being small, heat stable *Listeria*-active peptides that do not contain lanthionine (Klaenhammer, 1993, Nes et al., 1996). The heat stability of the bacteriocin was considered an advantage should the purified bacteriocin be used for biopreservation in a heat treated food. The bacteriocin was also shown to be stable at pH levels ranging from pH 2 to 9, which would make it suitable for use in meat (typical pH 5.5 to 6.0, Dainty and Mackey, 1992) as well as low pH fermented products. In order to classify and distinguish this bacteriocin from other bacteriocins, it was necessary to purify it to homogeneity and to determine the primary structure by amino acid sequencing.

Using hydrophobic interaction chromatography, cation exchange chromatography and reverse phase HPLC, enterocin B was purified and the amino acid sequence of the

mature peptide was elucidated by Edman degradation. At the time, this bacteriocin appeared to be novel and showed homology (47% identity) only to carnobacteriocin A. The bacteriocin was named enterocin 900 but it was renamed to enterocin B when Casaus et al. (1997) reported an identical amino acid sequence for the bacteriocin enterocin B produced by *E. faecium* T136. To date, enterocin B has homology only to carnobacteriocin A. These bacteriocins are similar to class IIa bacteriocins in that they are small, heat stable, do not contain lanthionine, and they are active against *Listeria monocytogenes*. However, unlike other class IIa bacteriocins they do not contain the conserved YGNGVXC consensus motif at their N-termini. The role of this YGNGVXC motif of class IIa bacteriocins is not understood. It has been suggested that this region could be involved in recognition of *Listeria* target membranes (Klaenhammer, 1993; Abee et al., 1995), but recently it was shown with phospholipid vesicles that electrostatic interactions of positively charged N-terminal residues and not the YGNGVXC consensus motif governs binding of pediocin PA-1 (Chen et al., 1997).

E. faecium BFE 900 does not harbor plasmid DNA, indicating that the genes responsible for bacteriocin production reside on the chromosome. The structural gene for enterocin B was localized on a 2.2-kb *Hin*dIII and a 12.0-kb *Eco*RI chromosomal fragment by using a 32-mer degenerate probe complementary to the 5' end of part of the gene encoding mature enterocin B. Both of these chromosomal fragments were fully sequenced in both directions and the enterocin B structural gene could be identified on both fragments. The structural gene encoded a prepeptide consisting of a double-glycine-type leader and the mature bacteriocin peptide, which is typical for the majority of class II bacteriocins (Klaenhammer et al., 1993; Nes et al., 1996). Unlike other class II bacteriocin systems the enterocin B immunity genes could not be located immediately downstream of the structural gene in an operon structure. This paralleled the case of carnobacteriocin A where the immunity was not located immediately downstream of the structural gene (Worobo et al., 1994). The absence of an immunity gene immediately following the bacteriocin gene and absence of a YGNGVXC consensus motif at the N-terminus of the bacteriocin are two characteristics that distinguish both enterocin B and carnobacteriocin A from other class IIa bacteriocins. This could be viewed as sufficient grounds for description of a further subdivision of class II bacteriocins. Accordingly class

IId could be provisionally classified as small, heat stable, nonlanthionine containing bacteriocins that have anti-*Listeria* activity but do not possess an N-terminal YGNGVXC consensus motif, that display homology in their carboxy termini, but do not exhibit tandem arrangement of bacteriocin structural and immunity genes.

Localization and identification of the immunity genes for both carnobacteriocin A and enterocin B represented an important aspect of this study. The enterocin B immunity gene (*eniB*) was located immediately downstream of, and in opposite orientation to the enterocin B structural gene. This gene conferred full immunity to the sensitive *L. sake* DSM 20017 heterologous host in immunity expression tests. The carnobacteriocin A immunity gene (*cbiA*) was also found downstream, but at a considerable distance (760 bp) from the carnobacteriocin A structural gene, and in the opposite orientation to the bacteriocin gene. The two immunity genes are similar in size (58 and 56 amino acids for EniB and CbiA, respectively) and both are chemically similar in that they have polar ends and a putative α-helical central core region. This region could insert into the membrane, and based on similarities of the immunity proteins it is postulated that they may act in a similar manner by inserting into the membrane. Membrane insertion may interfere with bacteriocin binding or pore formation, as previously suggested for other immunity proteins containing membrane spanning helices (Allison and Klaenhammer, 1996). In contrast to *eniB*, the *cbiA* gene conferred only partial immunity to the CbnA sensitive *C. piscicola* LV17C homologous host in immunity expression tests. The reason for this may be that bacteriocin production and immunity in the enterocin B system is constitutive, while in the carnobacteriocin A system it is regulated (discussed below).

Physical separation of the bacteriocin and immunity genes in the enterocin B and carnobacteriocin A systems represent two exceptional cases and it has prevented subcloning of the bacteriocin genes together with their respective immunity genes in heterologous hosts. Now that the immunity genes have been identified, heterologous expression of these bacteriocin and immunity genes, alone or in combination with other bacteriocin and immunity genes in a bacteriocin cassette is a distinct possibility (discussed below). The similarity between the enterocin B and carnobacteriocin A bacteriocin and immunity peptides suggests the further exciting possibility for exchange of the immunity genes in the two bacteriocin systems. Thus *eniB* may provide self-

protection to CbnA and *CbiA* may provide protection against EntB. Investigations of heterologous expression of the bacteriocins together with their own dedicated immunity genes (*entB* together with *eniB*, *cbnA* together with *cbiA*), and investigations of cross-protection by cloning of the immunity genes behind the different bacteriocin genes (*entB* together with *cbiA*, *cbnA* together with *eniB*) are well under way. Should cross-protection result, a further exciting avenue for research involves use of either of the immunity proteins as a 'food grade' selection marker. Lactacin F has been used previously to select for lactobacilli that were transformed with a recombinant plasmid containing the lactacin F immunity gene (Allison and Klaenhammer, 1996). The enterocin B or carnobacteriocin A immunity gene may be useful as a selection marker for enterococci or carnobacteria which contain a recombinant plasmid with the immunity gene.

Apart from the enterocin B structural and immunity genes no further genes associated with bacteriocin production could be identified on the 2.2-kb and 12.0-kb fragments. One possible exception was an ORF that showed characteristics of an induction factor (see below). Genetic evidence suggested that EntB was produced as a prepeptide bearing a leader peptide of the double-glycine-type. Accordingly this prepeptide would require a dedicated ABC transporter protein and an accessory protein, to remove the leader peptide and to transport the mature peptide out of the cell as is common for other class II bacteriocins that access these dedicated transport mechanisms (Håvarstein et al., 1995). The absence of such genes on the 12.0-kb fragment indicated that such genes could be located at a considerable distance (more than 9 kb upstream and 3 kb downstream) from *entB*. A probable explanation for this is that *E. faecium* BFE 900 produces more than one bacteriocin and that the transporter proteins of the second bacteriocin also serve to transport enterocin B. Evidence that *E. faecium* BFE 900 produces a second, 'pediocin-like' bacteriocin, enterocin A (previously described by Aymerich et al., 1996) was obtained. The mature part of this gene was amplified from chromosomal DNA by PCR using primers which corresponded to the 5' end of the mature part and the 3' end of the enterocin A gene as described by Aymerich et al. (1996). Production of enterocin A by *E. faecium* BFE 900 was confirmed by MALDI-TOF spectrometry, where a compound with a mass corresponding to that described for enterocin A (Aymerich et al., 1996) was detected in the supernatant of an overnight

culture. If enterocin B is transported using enterocin A transport proteins this would represent an unusual case, one that is only paralleled by the carnobacteriocin BM1 system. The carnobacteriocin BM1 structural and immunity genes are located on the chromosome, but the genes necessary for carnobacteriocin BM1 transport are the plasmid-encoded ABC transporter and accessory genes associated with production of carnobacteriocin B2 (Quadri et al., 1994).

For expression of bacteriocins in heterologous hosts the genetic determinants for bacteriocin production need to be identified and cloned, possibly on a recombinant plasmid. The smallest amounts of DNA required to encode production of, for example, pediocin PA-1 (Marugg et al., 1992) or lactococcin A (van Belkum et al., 1991) is 4 to 5 kb. Other bacteriocins, especially those whose production requires the presence of regulatory genes may require up to 10 kb of DNA (Diep et al., 1996; Hühne et al., 1996; Quadri et al., 1997). In contrast, the production of the divergicin A bacteriocin relies only on the structural and immunity genes, which amount to approximately 450 bp of DNA. This results from the fact that divergicin A is transported out of the cell by the *sec*-pathway also known as preprotein translocase (Economou, 1998) which relies on *sec* proteins for transport (Worobo et al., 1995). Accordingly, the structural gene of divergicin A is encoded as a prepeptide that bears a signal peptide sequence. The signal peptide directs transport to the preprotein translocase and is removed concomitant with transport (Worobo et al., 1995). Heterologous expression of carnobacteriocin B2 and colicin V has been achieved by fusing the part of their genes encoding the mature peptide to the divergicin A signal sequence (McCormick et al., 1996; McCormick, 1997).

This innovative possibility for secretion was used in this study to obtain production of enterocin B in the heterologous host *E. faecalis* ATCC 19433 by the *sec*-pathway. Secretion of enterocin B by its own, dedicated transport machinery was impossible, because genes for the dedicated ABC transport and accessory proteins were not present on the 2.2 or 12.0 kb fragments cloned from *E. faecium* BFE 900. The possibility for enterocin B transport by a heterologous host was also investigated. Earlier van Belkum et al. (1997) fused the leader peptides of leucocin A, lactococcin A and colicin V to the part of the gene encoding mature divergicin A and achieved expression of divergicin A in the respective homologous hosts. In some cases, divergicin production

was also observed when the leader peptides were used in heterologous hosts, indicating that the leader peptide directs secretion of bacteriocins by ABC transporters (van Belkum et al., 1997). A recombinant plasmid containing the 2.2-kb fragment of *E. faecium* BFE 900 was cloned into the carnobacteriocin A producer *C. piscicola* LV17A. This clone produced enterocin B, and it was concluded that the enterocin B leader peptide was recognized and correctly processed by the ABC transporter and accessory genes for carnobacteriocin A.

One problem associated with biopreservation of foods is the development of a bacteriocin-resistant population of cells after exposure to bacteriocin. The narrow inhibitory spectrum of some bacteriocins may also be a hurdle to use in preservation of foods. A suggested approach to improve the antimicrobial spectrum of an organism and to counteract resistance, is to develop gene cassettes for the production of multiple bacteriocins (McMullen and Stiles, 1996). Because a minimum of 4 to 5 kb of DNA is required for production of some bacteriocins (see above), it is difficult to envisage multiple bacteriocin production from a single vector. An elegant solution is to fuse bacteriocin structural genes with a signal peptide such as the divergicin A signal peptide. In that case approximately 0.5 bp of DNA would be required for production of a single bacteriocin. Such research is ongoing in our laboratory and production of the bacteriocins carnobacteriocin B2, brochocin C and colicin V by the *sec*-pathway has already been achieved. The cloning of carnobacteriocin B2 and leucocin A into a single, segregationally stable vector and production of these bacteriocins from a single organism is currently under investigation (Albert, 1998). Now that the immunity genes for both enterocin B and carnobacteriocin A are known, integration of these bacteriocins and their immunity proteins into such gene cassettes becomes a distinct possibility.

It has been reported that enterocins A and B act synergistically, i.e., the inhibition of the two bacteriocins acting together was greater than that of each bacteriocin acting alone. In this study we observed that the antimicrobial activity of bacteriocin produced by the wild-type strain was greater than that of enterocin B alone. For example, while the wild-type inactivated the indicator strains *Carnobacteriocin piscicola* UAL26 and LV17C, the enterocin B expressing heterologous *Enterococcus faecalis* ATCC 19433 (containing plasmid pCF03, see chapter 3) showed no activity towards these strains.

Therefore, inhibition of these indicators by the wild-type could be explained by the synergistic activity of enterocins A and B. A difference in inhibition spectra, as well as differences in levels of inhibition towards the same indicator strains was also noted by Casaus et al. (1997). Nevertheless both enterocins A and B showed antimicrobial activity against strains of *Lactobacillus* spp., *Enterococcus faecalis, Clostridium* spp., *Listeria monocytogenes* and *Staphylococcus aureus* (Casaus et al., 1997). Therefore, to obtain greater antimicrobial activity it would be best to clone both the enterocin A and B bacteriocin and immunity genes in a bacteriocin cassette, and for this the enterocin A structural and immunity genes would need to be isolated. Enterocin A is a 'pediocin-like' bacteriocin that contains the YGNGVXC consensus sequence at the N-terminus of the mature peptide. It would be interesting to determine whether enterocin B and other 'pediocin-like' bacteriocins such as carnobacteriocin B2 or leucocin A also show synergistic activity. In that case a greater antimicrobial activity could be obtained by including enterocin B together with these bacteriocins into a bacteriocin cassette. Results of Gao (1998) have shown that the partially purified bacteriocin fractions from the supernatants of *E. faecium* BFE 900 and the brochocin C producing *Brochothrix campestris* cultures act synergistically. It would be of great importance to determine if this synergistic activity could also be obtained from a combination of partially purified fractions from culture supernatants of the enterocin B producing *E. faecalis* ATCC 19433 heterologous host (containing pCF03) and *B. campestris*.

An intriguing observation was that enterocin B production by *Enterococcus faecium* strain BFE 900 was constitutive, while production of this bacteriocin in strain CTC 492 was regulated. The presence of a conserved, regulatory-type box was observed upstream of the *entB* promoter region in *Enterococcus faecium* BFE 900; unfortunately no genetic data is available for production of EntB in *E. faecium* CTC 492 (Nilsen et al., 1998). A conserved, regulatory-type box could not be deduced from the genetic data presented for EntB production in *E. faecium* T136 (Casaus et al., 1997). The presence of such regulatory sequences leads to the assumption that EntB in *E. faecium* BFE 900 is regulated; also the presence of an ORF with characteristics of an induction factor gene downstream of *entB* would support this assumption. Yet this is not the case. A possible explanation for constitutive EntB production would be that the –35 and –10 promoter

sequences located immediately downstream of the conserved regulatory-like box which are under the control of the transcriptional regulator are not utilized; instead alternative –35 and –10 promoter sequences located further downstream and closer to the ATG start codon for *entB* may be utilized for constitutive production of bacteriocin. Hypothetically, a mutational event occurred that changed the regulated bacteriocin phenotype to a constitutive production phenotype. An induction factor (EntF) was described for EntA and EntB production in *E. faecalis* CTC 492. The putative protein product of the ORF with characteristics of an induction factor gene in our study differs considerably from EntF. At present it is not known whether the putative induction factor of our study plays a role in EntB production or whether it can induce EntB production in *E. faecium* CTC 492. Investigations concerning the possibility that the putative induction factor encoded on the 2.2-kb fragment from *E. faecium* BFE 900 can induce bacteriocin production in *E. faecium* CTC 492 are currently under way.

The overall objective of this thesis has been met and all of the aims were fulfilled. A bacteriocin-producing *Enterococcus faecium* was isolated from food and the characteristics of its bacteriocin were determined. The bacteriocin is heat stable and active over a wide pH range, suggesting that it is a good candidate for use in foods. The bacteriocin was purified to homogeneity and the amino acid sequence was determined. The genetic determinants for bacteriocin production and immunity were identified and expressed in heterologous hosts. Unfortunately the same bacteriocin from another source was under investigation at a different laboratory concurrent with this investigation. However, the genes for enterocin B and carnobacteriocin A immunity proteins have not been identified before and this is a considerable extension of previously reported work. Also, interesting differences in regulation of enterocin B production were shown to exist in the strain used in our study. Elucidation of the genetic determinants for both enterocin B and carnobacteriocin A production will allow cloning of these bacteriocins into a multiple bacteriocin cassette. This represents considerable progress towards the ultimate goal of the various ongoing projects in our laboratory, i.e., to achieve multiple bacteriocin production by a single starter culture that will be used in the biopreservation of raw, modified atmosphere-packaged meat. Initial applied studies with single bacteriocin

producers (Leisner et al., 1996; Panayach, 1998) have successfully prolonged the shelf life of vacuum-packaged raw minced beef. This encourages the use of starter culture expressing multiple bacteriocins for achieving even better control over product spoilage. In addition, a starter culture that produces, among other bacteriocins, also an anti-*Enterococcus* bacteriocin would be of great advantage to control the dissemination of these potential pathogens.

5.1. References

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